HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC (HPLC) SEPARATION AND DETERMINATION OF FOUR VITAMINS IN SOME KENYAN FOODS

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A thesis submitted in partial fulfillment for the degree of Masters of Science of Kenyatta University

Nyambaka, Hudson N. High-performance liquid chromatography

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This thesis is my original work and has not been presented for a degree in any other University.

[Signature]

This thesis has been submitted for examination with my approval as University supervisor.

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Dr Gathu Nyagah
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DEDICATION

To

My parents

I wish to thank my dissertation committee, Dr. David Brown, for his careful direction, advice, and encouragement while preparing and conducting this work. His efforts in reading and evaluating the manuscript are well appreciated.

I am grateful to the members of the faculty and administration for their cooperation and assistance during my study. I cannot forget Ayse Tuncel for her initial assistance in using HPLC instruments. Also, I have benefited from all those who, in one way or the other, contributed to the success of this work.

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Techniques utilizing high-performance liquid chromatography (HPLC) were developed for the determination of four vitamins, β-carotene, α-tocopherol, thiamine and riboflavin, in some commonly consumed foods in Kenya. The techniques involve the simultaneous extraction and determination of β-carotene and α-tocopherol on one hand, and thiamine and riboflavin on the other hand.

The technique for the simultaneous determination of β-carotene and α-tocopherol involved sample saponification in an ethanolic alkaline solution and the extraction of the unsaponifiable matter with n-hexane. After evaporation of the solvent under vacuum, the residue was dissolved in ethanol and then assayed on a reversed phase HPLC system. The HPLC system used for the separation of the vitamins and various forms of carotene and tocopherol from each other as well as from ingredients in the sample matrix consisted of a µBondapack C_{18} reversed phase column and a mobile phase composed of methanol-acetonitrile-chloroform-water in the ratio 46:30:18:6. The vitamins were detected from their ultraviolet (UV) absorbance at 297nm.

The method was shown to be reproducible for both vitamins with coefficients of variation between 2.0 and 7.3%. The average recovery for β-carotene was 95.0%
and that for \( \alpha \)-tocopherol was 94.7%. Detection limits were 1.0 \( \mu \)g/ml and 2.0 \( \mu \)g/ml for \( \beta \)-carotene and \( \alpha \)-tocopherol respectively.

Thiamine and riboflavin determination involved extraction of the vitamins by hydrolysis in a limited amount of dilute hydrochloric acid followed by enzymatic incubation for 2.5 hours. Then the mixture was filtered and subsequently injected into a \( \mu \)Bondapak C\(_{18} \) reversed phase column without further cleanup. The mobile phase used for separation consisted of 0.01M phosphate buffer plus 1-hexanesulfonic acid sodium salt as counter ion (PH 7.0), methanol and acetonitrile in ratio 80:12:8. Detection of the vitamins was from their UV absorbance at 265nm.

The coefficients of variation of thiamine and riboflavin, between 4.4 and 9.0%, and 3.1 and 5.6% respectively, indicated that the method was reproducible. The average recoveries of thiamine and riboflavin were 92.0% and 93.7% respectively. Detection limits were 1.0 \( \mu \)g/ml for thiamine and 0.5 \( \mu \)g/ml for riboflavin.

The methods developed were applied to the analysis of some foods. The results indicate that indigenous green vegetables are good sources of \( \beta \)-carotene and \( \alpha \)-tocopherol in comparison to the exotic vegetables such as cabbage and lettuce. The levels of both \( \beta \)-carotene and \( \alpha \)-tocopherol in the indigenous green vegetables are between 3000 and 10650 \( \mu \)g per 100g.
of the vegetables, while those of exotic vegetables were between 30 to 230 µg per 100g.

Indigenous green vegetables were also found to contain high amounts of riboflavin. Legumes are good sources of thiamine and contain some riboflavin. They are, however, poor sources of β-carotene and α-tocopherol.
CHAPTER 1

INTRODUCTION

1.1. Description of Vitamins

Vitamins are biologically active organic compounds which are essential for an organism's normal health and growth. Organisms are unable to synthesize these compounds by anabolic process that are independent of the environment other than air\(^1\), and thus they must be obtained from the diet. In the body vitamins are required in small amounts since they play essentially catalytic roles in the normal metabolism of carbohydrates, fats and proteins to provide energy and regulate the building of body structure. The definition, however, tends to isolate some substances that are grouped under vitamins because they can be synthesized within the body of some species\(^2,3\), or because of the role they play in the body. Vitamin C (ascorbic acid), for example, can be synthesized in the body by most species of animals, except when they are young or under stress conditions. Vitamin D (cholecalciferol) is also synthesized in the skin of animals (including human) under the action of ultraviolet light. Similarly, some vitamins, such as
vitamin D act as prohormones in the body instead of acting as coenzymes. Under such conditions vitamins C and D are not regarded as vitamins.

The lack of a particular vitamin in the body causes deficiency diseases (avitaminosis) such as night blindness, beriberi, scurvy, rickets and pellagra (when vitamins A, B₁, C, D and niacin respectively are lacking). Though the diseases now known to be due to the lack of vitamins in the diet were recognized way back in the seventieth century and their empirical cures applied such as eating liver to cure night blindness, adding special substances to the diet to cure beriberi, and eating fruits and vegetables to cure scurvy⁴⁻⁵, the discovery of vitamins was only made in the first quarter of this century. The discovery came about through three different approaches during the study of accessory food factors: the study of diseases in man due to deficient diet, the discovery of diseases in animals similar to those in man, and the feeding of highly purified foodstuffs first to animals and later to man.

When Casimir Funk was studying the antiberiberi factor in 1912, he used the term *vitamine* for a compound that was essential to life and which he believed was an amine. However, the chemical nature of the essential compounds revealed later that most of
them are not amines. Hence the word vitamin, without the letter "e", was instead used to describe these essential compounds.

Before the chemical nature of vitamins was known, the naming system used was letter designation. This system however resulted into complications when later structural analysis revealed that some of the vitamin activity originally ascribed to a single compound depended on several compounds, such as vitamin B complex. Trivial names were thus adapted which are now commonly used and have been accepted internationally. However, some vitamins are still known by their letter designations.

Vitamins are present in minute quantities in many types of foods, both of animal and plant kingdoms. However no single food contains all the vitamins in sufficient quantities to satisfy the requirements under normal conditions of food intake\(^4\). Variation of the vitamin values in foods, especially vegetables and fruits, is caused by many factors such as variety and maturity of the tissues\(^6\). Growing conditions such as the weather, growing season, intensity of sunlight, and soil state also have an influence on the vitamin values\(^6-8\).
1.2. Classification and Properties of Vitamins

Although vitamins are diverse in their chemical characteristics, they can be classified into two groups on the basis of their solubility characteristics: the fat-soluble and the water-soluble vitamins. This classification is useful because it groups vitamins according to the problems associated with their chemical analysis and some common physiological characteristics.

Table 1.1 gives a list of the main vitamins according to their groups, together with some of their main sources. A short account of each group and the vitamins covered in this study is given below.
Table 1.1  List of some of the recognized vitamins and their main sources

<table>
<thead>
<tr>
<th>Letter designation</th>
<th>Commonly used names</th>
<th>Main sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat - soluble</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin A</td>
<td>Retinol</td>
<td>fish, cattle liver, eggs, milk</td>
</tr>
<tr>
<td>Provitamin A</td>
<td>Carotene</td>
<td>green leafy vegetables, carrots, potatoes, beans, fruits</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>Cholecalciferol and Ergocalciferol</td>
<td>liver, oils, egg yolk, liver, milk, green leafy vegetables</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>Tocopherol and Tocotrienol forms</td>
<td>seed oils, egg yolk, milk, liver, fruits, green leafy vegetables</td>
</tr>
<tr>
<td>Vitamin K</td>
<td>Phylloquinone (K(_1)), Menaquinone (K(_2)), and Menaphthone (K(_3))</td>
<td>fresh dark green vegetables, soya beans, tomatoes, lean meat, peas, carrots, milk</td>
</tr>
<tr>
<td>Water - soluble</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin B(_1)</td>
<td>Thiamine</td>
<td>lean meat, yeast, eggs, whole cereals, legumes, potatoes</td>
</tr>
<tr>
<td>Vitamin B(_2)</td>
<td>Riboflavin</td>
<td>yeast, milk, green leafy vegetables</td>
</tr>
<tr>
<td>Vitamin B(_5)</td>
<td>Pantothenic acid</td>
<td>organ meat, egg yolk, fresh vegetables</td>
</tr>
<tr>
<td>Vitamin B(_6)</td>
<td>Pyridoxine ((\text{Pyridoxal}, \text{Pyridoxal and Pyridoxamine}))</td>
<td>yeast, organ meat, fish, grains, cereals, potatoes</td>
</tr>
<tr>
<td>Vitamin B(_7)</td>
<td>Niacin ((\text{Nicotinic acid and Nicotinamide}))</td>
<td>yeast, organ meat, bran, fish, wheat, grains</td>
</tr>
<tr>
<td>Vitamin B(_{12})</td>
<td>Cyanocobalamin</td>
<td>mainly in animal liver, milk</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>Ascorbic acid</td>
<td>fruits, vegetables</td>
</tr>
<tr>
<td>Vitamin H</td>
<td>Biotin</td>
<td>organ meat, egg yolk, fresh vegetables</td>
</tr>
<tr>
<td>Vitamin M</td>
<td>Folic acid ((\text{Folacin}))</td>
<td>cereals, yeast, leafy vegetables</td>
</tr>
</tbody>
</table>
1.2.1. Fat - Soluble Vitamins

These vitamins are normally found in and extracted from food in association with lipids. In the body the vitamins are absorbed along with dietary fat. They are not normally excreted in urine but instead they are stored in the body. Most of the vitamins in this group are labile in the presence of oxygen or oxidizing agents as well as in light, especially ultraviolet light. But they are fairly stable to heat such that they do not decompose when cooking or processing.

1.2.1.1. \( \beta \) - Carotene

\( \beta \)-Carotene is one of the various forms of the naturally occurring carotenoids which are found in plants and have vitamin A activity (provitamin A compounds). Other carotenoids that have fairly high vitamin A activity include \( \alpha \)-, \( \gamma \)-carotenes, \( \beta \)-cryptoxanthin and 9- and 15-cis isomers of \( \beta \)-carotene. These provitamin A carotenoids form the main source of vitamin A. They are produced by plants and converted metabolically to vitamin A by animals.

Of the provitamin A carotenoids, \( \beta \)-carotene is the most widely distributed in nature. It exists in large amounts in plant foods and has the highest vitamin A activity. It is thus regarded as the most important nutritionally.
$\beta$-Carotene is easily oxidized by air or oxygen, a process that is stimulated by light and fat peroxides. Since fat peroxides oxidize $\beta$-carotene, conditions that favour oxidation of fats will also oxidize it.

The chemical oxidation of $\beta$-carotene gives a 5,6-epoxide, while light catalyzed oxidation yields mutachrome. At higher temperatures $\beta$-carotene can fragment to give a series of aromatic hydrocarbons, the most important being ionone. This instability of $\beta$-carotene is accelerated in acid conditions but greatly reduced in alkali media.
1.2.1.2. **α-Tocopherol**

α-Tocopherol is one of the eight naturally occurring forms of vitamin E that are closely related biologically. These forms of vitamin E are divided into tocopherols and tocotrienols which differ by the fact that tocotrienols possess three double bonds in the isoprenoid sidechain. The individual forms in each group arise from the substitution of one or more methyl groups on the ring system at the 5-, 7- or 8-positions, as shown in Table 1.2 below:

<table>
<thead>
<tr>
<th>Substitution</th>
<th>Tocopherol</th>
<th>Tocotrienol</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-methyl</td>
<td>6-Tocopherol(6T); 6-Tocotrienol(63T)</td>
<td></td>
</tr>
<tr>
<td>5,8-dimethyl</td>
<td>5-Tocopherol(5T); 5-Tocotrienol(53T)</td>
<td></td>
</tr>
<tr>
<td>7,8-dimethyl</td>
<td>7-Tocopherol(7T); 7-Tocotrienol(73T)</td>
<td></td>
</tr>
<tr>
<td>5,7,8-trimethyl</td>
<td>5-Tocopherol(5T); 5-Tocotrienol(53T)</td>
<td></td>
</tr>
</tbody>
</table>

**α-Tocopherol** (the form in which all positions are substituted by the methyl group) is the most active and widely distributed of the naturally occurring vitamin E compounds. Other compounds have 30 or less percent of α-tocopherol potency.
\[ \text{HO} \]

\[ \begin{array}{c}
\text{Figure 1.2 The structure of } \alpha\text{-Tocopherol} \\
\end{array} \]

\( \alpha\text{-Tocopherol} \) is readily oxidized by the atmospheric oxygen, a process which is rapidly increased by heat and catalyzed by metal salts of iron, zinc or silver, and unsaturated fatty acids. The decomposition products of oxidized tocopherols include dimers, trimers, dihydroxy compounds and quinones. \( \alpha\text{-Tocopherol} \) is stable to heat, acids and alkalis in the absence of oxygen or oxidizing agents, but unstable to alkali in their presence.

1.2.2. Water-Soluble Vitamins

Water-soluble vitamins are not stored in any appreciable amounts in the body and any excess is excreted in the urine. These vitamins show wide variation in their reaction properties. Some of the
vitamins, for example, are stable to heat such as riboflavin and pyridoxine, while others are heat labile such as thiamine and ascorbic acid.

Most of the water-soluble vitamins are labile to light, especially ultraviolet light, and are easily oxidized (thiamine is oxidized to thiochrome while riboflavin decomposes). However, most of them are stable under acid conditions.

1.2.2.1. Thiamine

Thiamine (vitamin B\textsubscript{1}) is widely distributed throughout the plant and animal foods, with the main sources given in Table 1.1. It can exist either as free thiamine or as one of its phosphate esters (thiamine monophosphate (TMP), thiamine diphosphate (TDP) and thiamine triphosphate (TTP)) that are bound to the respective apoenzyme. In plants, the vitamin mainly occurs as free thiamine. Thiamine plays a key role as a coenzyme in the intermediary metabolism of \( \alpha \)-keto acids and carbohydrates.

Since thiamine nucleus is a cation, it forms salts with many acids. The structure of thiamine hydrochloride is given in Figure 1.3.
Figure 1.3 The structure of Thiamine Hydrochloride

Thiamine is one of the least stable vitamins, especially in alkaline or neutral solution due probably to the thiazole ring which opens and is rapidly oxidized upon heating. The stability of thiamine is also affected by many factors such as temperature and some reacting species.

Thiamine is easily oxidized by a variety of oxidizing agents, including oxygen, to the biologically inactive thiochrome. It can also be reduced irreversibly to a dihydro form which is inactive. These processes are greatly accelerated in alkaline solution but retarded in acid media.

Strong nucleophiles such as sulphites and neutral or alkaline solutions cause nucleophilic displacement of thiamine at the methylene carbon joining the two rings to give two derivatives; 5-β-hydroxyethyl-4-
methythiazole and a corresponding substituted pyrimidine. In the case of sulphites, the substituted pyrimidine is 2-methy-5(methysulfonic acid)pyrimidine. However, sulphite reactions can be retarded by the use of casein or soluble starch.

The nitrites, on the other hand, inactivates thiamine via a reaction with an amino group on the pyrimidine ring. But the proteins found in foods protect the vitamin against this action.

At high temperatures, thiamine easily decomposes and leads to rearrangement and decomposition of the thiazole ring resulting into various products such as elemental sulphur, hydrogen, sulphide, furan, thiophene and dihydrothiophene. At 100°C for example, thiamine can degrade at a rate ranging from 0.0020 to 0.0027 per minute.

### 1.2.2.2. Riboflavin

Riboflavin usually occurs in foods either in the free form or as riboflavin 5-phosphate, i.e. flavin mononucleotide (FMN), or flavin adenine dinucleotide (FAD). The principal forms of riboflavin in nature are FMN and FAD, with the latter occurring in greatest abundance (69-90%). In natural products these forms of the vitamin are combined with certain proteins to form catalytically active flavoproteins which play an important role in tissue oxidation essential for normal growth (such as in the citric acid cycle).
Figure 1.4 The structure of Riboflavin

Riboflavin is easily degraded by visible or ultraviolet (uv) light in solution, but it is relatively stable in dry form under normal light. In alkaline solution, visible or uv irradiation degrades riboflavin at the side chain to form lumiflavin, whereas in neutral or acidic media the same treatment gives the blue fluorescent substance, lumichrome.

Riboflavin is resistant to most oxidizing agents, especially in acid media. It is also heat-stable in acid solution. But it is reduced by dithionite, Zn/H⁺, TlCl₃ or mild catalytic hydrogenation to yield the hydroquinone. When reduced by conventional methods (water, sodium hydrosulphite) riboflavin readily takes up 2 hydrogen atoms, giving rise to colourless and
nonfluorescent 1,10-dihydro compound (leucoriboflavin). This compound can be reoxidized to the vitamin by shaking an aqueous suspension with air.

1.3. Deficiency Effects of Vitamins

The lack of a particular vitamin causes a special disease which can only be cured by the intake of that vitamin. Vitamin deficiency diseases usually coexist with Protein-Energy malnutrition. The latter has been classified as one of the most common nutritional problems in the developing countries\textsuperscript{10,11}.

The initial manifestation of vitamin A deficiency is night blindness. Since vitamin A can be stored in the body of healthy adults, night blindness is rare in adults. It occurs mainly in children because they have lower vitamin A reserves in the body.

The other major vitamin A deficiency symptoms or diseases are xerophthalmia (drying of the cornea giving dull appearance of the eye), keratomelacia and increased risk of diarrhoea. Besides, there are minor changes like skin lesion and Bitot's spots in the eye that come about due to vitamin A deficiency. If xerophthalmia is not treated in time, it will lead to keratomalacia which causes blindness\textsuperscript{12}. Skin lesions may occur particularly if the individual is deficient in B complex vitamins as well.
According to a study done on the consequence of vitamin A deficiency in rural Indonesian children, there is increased risk of respiratory diseases and diarrhoea in children who suffer mild vitamin A deficiency\textsuperscript{13}. It has been established that diarrhoea is a major killer of children in poor countries of the world.

Recent research has shown that some provitamin A compounds have anticancer, antiaging and antiulcer properties\textsuperscript{14,16}. Epidemiological studies have shown the existence of an inverse relationship between the risk of cancer and the consumption of foods containing $\beta$-carotene\textsuperscript{15,16}. Also, several laboratory experiments have demonstrated the inhibition of cancer cell lines and actual tumor regression in animals given $\beta$-carotene\textsuperscript{16}.

For the antiulcer properties, it has been shown that $\beta$-carotene and $\beta$-cryptoxanthin are involved in the cytoprotective injury of the gastric mucosa\textsuperscript{14}. Similarly, the existence of the relationship between the concentration of carotenoids in serum and brain tissue with the maximal life span potential of mammalian species demonstrates the antiaging effects of carotenoids.

Vitamins E, the antisterility factor, is required for the normal maintenance of reproductive processes in animals. These properties, though, have not been proven in man and remains obscure. However, vitamin E is an
essential nutrient for humans. Some evidence of
deficiency is seen in children with fat absorption
defects (e.g. sprue, fibrocystic disease of the
pancreas). Similarly, creatinuria, ceroid pigmentation
and abnormal red cell haemolysis are encountered. It
has also been observed that vitamin E makes animals
resistant to cancer\textsuperscript{14}.

Apart from being nature's best fat-soluble
antioxidant, vitamin E appears to be critically
required for the maintenance of health and integrity of
every cell of the animal body. It functions as an
intracellular antioxidant and as such it helps prevent
oxidation of unsaturated fatty acids and vitamin A. It
helps to enhance the activity of vitamin A by
preventing its oxidation and loss in the intestinal
tract.

In one or more specific roles, vitamin E is
closely involved with the metabolism of selenium and
sulphur amino acids. The major function of vitamin E in
the body appears to reside in the protection of
phospholipids of the mitochondrial and microsomal
membranes from free-radical attack. If free radical
formation is not checked by adequate vitamin E and
selenium nutrition, it leads to peroxidation and
destruction of organelles which are important in the
production of antibodies and of other mechanisms
required for the normal recovery from pathological
diseases and other environmental stresses\textsuperscript{17}. Hence
vitamin E is needed for the optimum functioning of the normal body mechanism involved in defence against all diseases. It is clear, then, that vitamin E is involved in maintaining the health of the brain, the vascular system, the erythrocytes, the skeletal muscles, the liver and the heart.

It has been reported that the intake of vitamin E is needed with vitamin A for prevention of blindness, as well as other nutrients\textsuperscript{16}. It was found that malnourished children with xerophthalmia had significantly lower serum vitamin E levels than malnourished children without ocular lesions\textsuperscript{16}. There is also a correlation between the amount of vitamin E required and the intake of fat and polyunsaturated fatty acids. Increased intake of the latter compounds correspond to an increase in need for vitamin E\textsuperscript{17}.

Deficiency of thiamine in the body causes beriberi, a disease that ultimately affects the nervous system. Thiamine deficiency in man is characterized by accumulation of pyruvic and lactic acids in the blood and brain, and the impairment of cardiovascular, nervous and gastrointestinal functions. This results in the loss of appetite and the development of nausea and later to a form of neuritis and cardiac difficulties.

Riboflavin deficiency in man is associated with deficiency of other B complex vitamins which could result in a variety of problems. The importance of riboflavin in nutrition stems from its reaction with
protein to form flavoprotein enzymes that participate in a wide variety of metabolic reactions. Riboflavin (with vitamin B\textsubscript{2}) is involved in the conversion of tryptophan metabolism. Tryptophan is a naturally occurring amino acid which is essential for optimal growth in infants and for a proper nitrogen balance in adults. The body converts tryptophan to niacin, another vitamin in the B-group, whose deficiency in the body causes pellagra. Riboflavin deficiency in man is responsible for glossitis (inflammation) of the tongue and lips, scaliness at the corners of the mouth (cheilosis) and also causes impairment of the liver functioning. Changes in the eye which can involve corneal vascularization are also symptoms of riboflavin deficiency.

1.4. Vitamin Deficiency Problems in Kenya

The deficiency of vitamins is a major problem in developing countries. In Kenya the situation is made complex by the co-existence of the deficiency of other nutrients. For example, Protein-Energy deficiency together with the deficiency of vitamins has been reported\textsuperscript{10}. Similarly, the existence of the deficiency of essential minerals together with the deficiency of vitamins is very common\textsuperscript{10,18}. Vitamin deficiency in Kenya has been identified through field nutrition surveys and clinical research. As demonstrated both by dietary surveys and the
supporting clinical evidence vitamin A deficiency is a common nutritional problem in Kenya\textsuperscript{10,18,20}. However, some symptoms of this vitamin deficiency can go unnoticed due to the complex interaction of infection with various types of malnutrition and deficiency diseases. This can be exemplified by the reported xerophthalmic complex observed in some children with measles in Meru\textsuperscript{18}. Bohdal et al.\textsuperscript{10} conducted a nutrition survey in various parts of Kenya covering some locations in Nyeri, Muranga, Nyanza, Machakos, and Kitui and estimated vitamin A intake in diets to be below 30\% of the recommended in all areas surveyed. In Nyeri and Mwea-Tabere the intake of vitamin A was estimated to be 28\% and 8.1\% respectively\textsuperscript{20,21}.

Due to the fact that Vitamin E does not appear to be a specific cure for any disease in human except hemolytic anemias of premature infants\textsuperscript{16,22}, tocopherol has received little attention in nutritional deficiency studies. However, tocopherol deficiency has been reported in East Africa\textsuperscript{2}.

Nutritional deficiencies of riboflavin and thiamine have both been reported in many parts of Kenya\textsuperscript{18,20,34}. However, deficiency of thiamine is rare in some places. Such adequacy of thiamine may be explained partly by the fact that cereals and legumes which are rich sources of thiamine, are consumed in most parts of the country\textsuperscript{20,21,23}. A nutritional survey conducted in Mwea-Tabere settlement revealed that
riboflavin intake in the area was well below the recommended levels and clinical evidence showed some signs of deficiency. Riboflavin intake of diets was approximated to be less than 70%. The same study found that intake of thiamine was above average. This was attributed to the constant consumption of rice which is a good source of thiamine.

Riboflavin deficiency problems have been also diagnosed in Machakos District and the areas bordering it.

1.5. Campaign against Vitamin Deficiency Problems

In developing countries the requirements of vitamins is mainly obtained from plant foods. Vegetables and fruits are good sources of β-carotene, α-tocopherol and riboflavin, while legumes are rich in B vitamins but lack riboflavin. Nutritional surveys carried out in Kenya have confirmed that a large proportion of the Kenyan population subsists on vegetarian diets. Some areas have staple diet of maize and beans. This situation can be accounted for, partly at least, by the fact that animal products which are good sources of most vitamins are not available or are too expensive to most people. In some areas such as the dry regions, dietary deficiency of vitamins can be attributed to the unavailability of green leafy vegetables, and seasonal unavailability in the periods of drought.
In the light of the vitamin deficiency problems in Kenya the need to educate the general public and decision makers on the advantage of alleviating vitamin deficiency has taken various approaches. In an effort to improve production and consumption of foods for better health, farmers have been encouraged to grow more nutritious foodstuffs such as legumes and indigenous cereals and vegetables. Such an effort is also noticed in articles that appear occasionally in local newspapers highlighting some of the local nutritious crops.

Nutritional researchers have also joined the campaign to alleviate nutritional deficiency problems. Some work has been reported on the storage and preservation techniques for some seasonal foods such as green leafy vegetables that are not available during dry seasons. Some international bodies have also initiated programs that encourage the general public to be self-reliant on the production of cheap nutritious foods. One such body is the United States Agency for International Development (USAID) which has a project for the promotion of household and community gardens with an aim of alleviating vitamin A deficiency in Africa by increasing vegetables, fruits and other family foods rich in vitamin A.
CHAPTER 2

LITERATURE SURVEY

2.1. Methods Commonly Used in the Analysis of Vitamins

The methods available in the literature for determination of vitamins can be classified into biological, microbiological and chemical (or physico-chemical) methods. Some of these methods have been accepted by the Association of Official Analytical Chemists (AOAC) as official methods for vitamin analysis\textsuperscript{35}. However, the selection of any method for analysis is based on its availability and applicability to the intended purpose\textsuperscript{36}. Some of the methods commonly used for the determination of vitamins under study are briefly reviewed below.

2.1.1. Biological or Bioassay Methods

The development of the present knowledge on vitamins is based on the assays with animals where the potencies of natural sources were compared in biological assays. Their purpose has often been to test whether particular preparations could cure deficiency symptoms.

Biological methods for vitamin assay are based on the biological effects using animal experiments, such as the curative tests on rats or the growth of chicks.
The measurement rely on the response in animals in terms of growth reaction time or the degree of variation with the vitamin dosage. The assay depends on an adequate basal ration that is complete in all essential factors except the vitamin under test.

Biological methods are important because vitamins are fundamentally concerned with animal and human nutrition. For the purpose of the availability of vitamins in foods, the direct test with animals gives essential information as to how much of the vitamin present is effective. However, the physiological availability is not always the same for different animal species. This is due to the complication of the intestinal synthesis of vitamins in animals including humans.

There are various biological assay procedures that are reported for the determination of thiamine and riboflavin, α-tocopherol, vitamin A and β-carotene. The procedures have the advantages of supplying an accurate estimation of biopotency without elaborate separation of the components. But since the methods depend on the animal response quantification is very difficult. The methods, also, require special animal facilities and usually take a long time, normally weeks, to carry out. Development of other methods that are less time consuming and reliable has therefore been a priority to most analysts.
2.1.2. Microbiological Methods

Microbiological techniques for the determination of vitamins are based on the fact that certain microorganisms require specific vitamins for growth. If a basal medium complete in all aspects except for the vitamin under test is used, the growth response of the organism in the standard and the unknown solutions can be compared quantitatively.

The microorganisms that can be used in these methods are bacteria, yeast or molds that are known to require external source of the vitamin. But the lactic acid bacteria group are the most widely used microorganisms because production of lactic acid enables growth of the organisms to be measured by titration or by pH change. Other ways of determining the extent of growth of the organisms include measuring the turbidity of the medium, drying and weighing the organism, measuring the nitrogen content of the cells or counting the actual cells.

Many microbiological procedures for analysis of vitamins have been reported, including those that are accepted as AOAC official methods. These methods are advantageous in that they provide an accurate measure of biopotency because the growth response of a particular microorganism is specific to a particular vitamin. However, the methods require special equipments, and analysts who have working
knowledge of bacteriological techniques and a certain measure of microbiological intuition. This makes the microbiological methods very expensive.

There are further disadvantages of microbiological methods for vitamin assay. Growth of some organisms, especially *lactobacilli*, is stimulated by some substances such as fatty acids and inhibited by anti-microbial substances like preservatives and antibiotics that are found in the basal medium but are non-chemically defined. This gives poorly reproducible and hard to standardize result. Also, since maximum growth of most organisms takes about 60-72 hours, the procedure becomes time consuming and tedious.

2.1.3. Physico-Chemical Methods

2.1.3.1. Colorimetric Method

The colorimetric methods are based on the formation of colour when a reagent has been added to a sample extract. The production of colour, which demonstrates the presence of a vitamin, has been exploited in many procedures of vitamin analysis, including the AOAC methods.

In the analysis of vitamin E the colorimetric method is known as Emmeric-Engel reaction. It is based on the fact that when *α*-tocopherol reduces ferric to ferrous iron in the presence of *α*, *α*-dipyridyl, a red complex is formed between the ferrous ions and *α*, *α*—
dipyridyl. The red complex then may be readily measured in a colorimeter or spectrophotometer. Other reagents used for the formation of colour have been reported. They include 4-amino-N,N-dimethylaniline, dipheny-(batho)phenanthroline, terpyridyl and 2,4,6-tripyrrolidyl-5-triazine.\textsuperscript{38}

The fact that other reducing agents such as carotenoids, sterols and phenolic compounds in plants also react with the reagents, especially $\alpha$, $\alpha$-dipyridyl, makes the method lack specificity. Most of the procedures given for the analysis of the vitamin by this method include a purification step that removes interfering compounds. The purification procedures mostly used are column chromatography\textsuperscript{36,38} paper chromatography\textsuperscript{38} and thin-layer chromatography\textsuperscript{38}. However, these procedures are characterised by loss of the vitamin by degradation since it is exposed to air and ultraviolet light. The method also involves long sample preparation and detection times.\textsuperscript{42}

In the determination of $\alpha$-tocopherol by the colorimetric method, the colour is not completely stable and blank readings tend to be high. Although some workers have used acetic acid to make the reaction colour more stable and produce a linear relationship with the amount of $\alpha$-tocopherol, the acid is corrosive to most instruments.\textsuperscript{38}
A number of chemical reagents produce coloured products with both preformed vitamin A and $\beta$-carotene, thus enabling many colorimetric procedures to be developed. But the classical Carr-Price method is popular and has found widespread application, including the AOAC methods, in the analysis of preformed vitamin A and $\beta$-carotene. The Carr-Price procedure relies on the formation of a blue coloured complex produced by the reaction of vitamin A or $\beta$-carotene with antimony trichloride ($\text{SbCl}_3$) in chloroform.

Although the method has been found to be reasonably reproducible, there is interference from the compounds including several decomposition products such as inactive isomeric forms of vitamin A, and other carotenoids$^{43,44}$. The compounds produce a similar colour, probably because the reagents convert them all to a common intermediate$^{44}$.

Apart from the unspecificity, Carr-Price procedure has problems of the developed colour being transient and the use of unpleasant corrosive reagents. In the measurement of $\beta$-carotene the maximum blue colour is reached after 5 seconds, after which it drops quite rapidly$^{40}$. This requires that measurement must be carried out during the short interval, necessitating the reaction to be carried out directly in the spectrophotometer.
A colorimetric method has been used to determine thiamine\textsuperscript{35}. The method involves the formation of a coloured pigment (usually red) when thiamine is allowed to react in alkaline medium with a diazotized aromatic amine, such as p-aminoacetophenone. However the method requires high concentrations of thiamine in samples.

2.1.3.2. Fluorimetric Methods

Fluorimetric methods utilize the fact that some vitamins fluoresces strongly when exposed to light of a particular wavelength. The intensity of the fluorescence is proportional to the amount of the vitamin in the sample being examined. With the development of commercial filter fluorometers and spectrophotofluorometers, attempts have been made to use the fluorescence properties of vitamins for their measurement.

The determination of thiamine by the fluorimetric methods is sometimes referred to as thiochrome methods. Various fluorimetric procedures have been reported including the official AOAC methods, for the determination of thiamine in foods\textsuperscript{4,36}. The methods depend upon the alkaline oxidation, using for example potassium ferrocynide, of thiamine to thiochrome which exhibits an intense blue fluorescence and is measured fluorimetrically.
The oxidation step in the fluorimetric technique for thiamine determination acts as a purification step that increases sensitivity, but some disulfide is also formed under these conditions\textsuperscript{37}. Presence of nonthiamine fluorescent substances in the sample affects the measurement. The time allowed for the generation of thiochrome during oxidation also affects the results\textsuperscript{35}.

The fluorimetric methods for the determination of riboflavin can be done either directly where free riboflavin and bound riboflavin (FMN and FAD) give a characteristic green-yellow fluorescence in aqueous solution\textsuperscript{35-37}; or indirectly by lumiflavin procedure\textsuperscript{37}. The lumiflavin procedure depends on the measurement of lumiflavin as a decomposition product that forms on irradiation of riboflavin in alkaline media.

The major drawback in the fluorimetric procedures for riboflavin determination is that riboflavin fluorescence is affected by visible light, various pigments, and anions such as halides, cyanides, thiocyanides, sulfites and nitrites\textsuperscript{37}. In addition a variety of other interfering fluorescent materials are often present in extracts of natural foods. But some procedures have been reported for elimination of interfering substances. These include the addition of stannous chloride, sodium hydrosulfite and permanganate solution to reduce the pigments\textsuperscript{35,37}. Similarly,
adsorption techniques are used to remove interfering substances. However, these additional manipulations are complex and time consuming.

Few fluorimetric procedures have been reported on the analysis of fat-soluble vitamins in foods, especially for \( \alpha \)-tocopherol and \( \beta \)-carotene. Isolated cases in the literature give procedures for the determination of vitamin A and tocopherols in biological samples\(^3\). Such procedures experience interference from phenolic estrogens, carotenoids and pentaene phytofluene\(^4\).

The use of fluorescence detectors in modern liquid chromatography has seen a wide application of fluorimetric procedures in the determination of vitamins in foods\(^8,45-48\). This has come about because fluorescence methods are usually said to be more specific than those based on absorbance. However, although the use of fluorometric detectors improves selectivity, fluorescence readings are affected by many factors, such as lamp output, detector sensitivity and the presence of quenching or enhancing factors\(^4\). This makes quantification difficult to control and requires that the instrument be calibrated frequently. Thus fluorimetric procedures are not entirely specific.

2.1.3.3. Spectrophotometric Methods

The Spectrophotometric procedures are based on the principle that some vitamins contain conjugated
double bonds which are associated with absorption of ultraviolet (UV) and visible light. The spectra of such vitamins display maxima absorbance at certain wavelengths. The UV absorbance has been used in quantitative analysis of vitamins for over 50 years, with an early attempt to standardize the technique made in 1934.4

Various spectrophotometric procedures for the determination of provitamin A and vitamin E have been reported4,36,38. However since some vitamins have low UV absorption and there are many interfering substances in foods, this method has found limited use. For example, the low intensity of absorption of its maximum ($E_{1cm}^1 = 72$ at 292 nm) has limited the usefulness of spectrophotometry in the measurement of $\alpha$-tocopherol38. In an effort to increase the low intensity of absorption, some workers have converted $\alpha$-tocopherol to the corresponding quinone which has much stronger absorbance5.

Since food samples contain many interfering substances such as carotenoids, sterols and a considerable quantity of mostly unknown reducing substances, extremely vigorous purification is required before use of this method on natural products. Purification procedures such as column chromatography, paper chromatography and thin-layer chromatography have been used. These procedures however, have not proved suitable for quantitative analysis because the vitamins
are destroyed by exposure to air and ultraviolet light. Recoveries are also low when these purification procedures are applied.

The development of modern liquid chromatographic systems with ultraviolet light absorbance detectors has enabled the development of procedures employing UV absorbance technique for vitamins analysis in foods.

2.1.3.4. Other Methods

There are other analytical methods appearing in the literature which are used for the determination of vitamins, especially vitamin E as \( \alpha \)-tocopherol. Some of the methods for determination of \( \alpha \)-tocopherol include titrimetry\(^3^8\), polarimetry\(^3^6\) and gas liquid chromatography (GLC)\(^3^6,^3^8\).

Titration with ceric sulphate has found particular application in the determination of high purity \( \alpha \)-tocopherol. Apart from the fact that the method is used for pure samples, thus requiring vigorous purification procedures in case of other samples, it is not used for low potency forms of tocopherol because of insufficient sensitivity.

Polarimetric methods of tocopherol determination utilize the fact that some forms of the vitamin are optically active. Measurement of their optical rotation has been used in various polarimetric procedures, including AOAC methods. These methods have been used for the identification of some optically active isomers.
of tocopherol in foods and pharmaceutical drugs\textsuperscript{36,38}. However, the procedures used for the separation and purification of the various tocopherols results in high losses of the vitamin through oxidation.

Gas liquid chromatographic methods have limited use in the analysis of vitamins. This may be explained by the fact that high temperatures are used for separation, a process that destroys most of the vitamins\textsuperscript{44}. But some GLC procedures have been developed for the determination of $\alpha$-tocopherol\textsuperscript{38} and thiamine\textsuperscript{49,50} in foods.

2.2. High Performance Liquid Chromatography (HPLC) and the Analysis of Vitamins

2.2.1. Introduction

The improvements in equipments, materials, techniques and the application of theory on the earlier developed chromatographic methods (column, thin-layer, paper and gas chromatography) led to the development of HPLC. The method offers major advantages in convenience, accuracy, speed and the ability to carry out difficult separation. HPLC may be considered as complementary to gas chromatography (GC) since both techniques may be used to effect the same separations. But compared to GC, HPLC makes difficult separations possible due to the use of two chromatographic phases, lower separation temperature and the availability of many unique columns. Also, HPLC is superior to GC
because samples analyzed are not limited by sample volatility or thermal stability, and because various detectors are available.

Since HPLC may operate at ambient temperature, has greater variety of unique column packings and has two chromatographic phases for selective interaction of molecules, it has been used widely for difficult separation such as macromolecules and ionic species of biomedical interest, labile natural products and a wide variety of other high-molecular weights and/or less stable compounds such as vitamins. Though only about 20 years old, HPLC has been applied in many fields including pharmaceutical, biochemical, clinical and environmental analysis.

There are four basic types of HPLC systems which differ in the unique nature of separation of the sample molecules in each: liquid-solid chromatography (LSC), ion-exchange chromatography, exclusion chromatography and partition or liquid-liquid chromatography (LLC). Liquid-solid chromatography depends upon the adsorption of the solute on polar adsorbents such as silica gel or alumina which use surface hydroxyl groups and Lewis acid type interactions for adsorption. This technique is widely used in such analysis as of pesticides, aflatoxins, estrogen steroids and vitamins.

Ion-exchange chromatography relies upon the exchange of the ions between the mobile phase and the stationary phase. The stationary phase consists of
polystyrene, silica or glass packing support which have various types of ion-exchangers chemically bonded. The mobile phase contains exchangeable counter ions to keep neutrality. During separation, the sample ions exchange with the counter ions. The technique has found widespread application in biological science particularly in the analysis of amino acids.

Exclusion or gel permeation chromatography has separation based upon the molecular sizes of the solute. The stationary phase consists of a gel packing with an inert porous surface such that during separation small molecules enter the network and larger ones pass through unretained. This technique is applicable to a wide range of material covering both high and low molecular weights. It has been used mainly for fractionation of polymers.

Partition or liquid-liquid chromatography (LLC) depends upon the partitioning of a solute between two immiscible solvents; the stationary phase and the mobile phase. The earlier polar or non-polar stationary phases, like in gas liquid chromatography, were coated onto an inert support and packed into the column. This method was found ineffective because the stationary phase was being removed after pumping large volumes of mobile phase. Similarly the fact that there are large changes in mobile phase composition, especially in gradient elution, limits the use of such stationary phase.
The need for more durable columns led to the development of bonded phase chromatography (BPC) where the stationary phase is chemically bound to the inert support. The ability to chemically bind a wide variety of materials to a rigid support has resulted in a large range of stationary phases with different polarities and selectivities; thus minimizing chromatographic problems experienced in coated packings. LLC is termed "normal phase" if the stationary phase is more polar than the mobile phase, and "reversed phase" if the mobile phase is more polar than the stationary phase.

The normal phase LLC has the packings bonded with polar groups and requires solvents identical with those used in liquid-solid chromatography. Some examples of polar packings are those with alkynitrile \((\text{Si-CH}_2)_n\text{CN}\) and alkylamine \((\text{Si-(CH}_2)_n\text{NH}_2)\) groups bonded to the inert surface through Si-O-Si bonds. Such columns require typical solvent mixtures of hydrocarbons, methylene chloride and tetrahydrofuran. However, these columns might be affected by the reaction between the functional groups of the bonded phase and compounds in the mobile phase or sample. For example, alkylamine bonded phases are subject to oxidation, and therefore strong oxidants should be avoided.

The reversed phase chromatography use a polar eluant such as water, methanol and/or acetonitrile with a non-polar stationary phase such as a hydrocarbon
bonded to an inert support. An example of such a stationary phase is octadecylsilyle-silica (ODS-silica) with the structure shown below

Though this method is commonly used for separation of polar molecules, current literature testifies that the reversed phase HPLC is used throughout a broad polarity range and in diverse applications. Reversed phase HPLC is a popular mode of analysis and it has been estimated that 60-80% of all HPLC separation are accomplished using reversed phase packings⁵².
2.2.2. HPLC Instrumental Principles

The fundamental components of a typical chromatograph are shown in Figure 2.1 in form of a block diagram.

![Figure 2.1 HPLC block diagram](image)

The solvent reservoir contains the mobile phase from where it is pumped through the feed line. The feed line is fitted with a filter to prevent particulates from being drawn into the pump and cause clogging.
The gradient device is used for changing the mobile phase composition during the analysis. Normally HPLC systems are classified into isocratic system where the mobile phase composition is kept constant, and gradient system where the mobile phase composition is continuously changed during the analysis. Gradient elution can be able to separate complex mixtures having widely varying capacity factors.

To overcome the resistance offered by the small particles used to pack columns, high pressure is required. This is achieved with the aid of a pump which also provides a constant reproducible supply of mobile phase to the column. There are two types of pumping systems available: those that deliver essentially constant flow rate, and those that deliver a constant pressure. Apart from being impractical to use in gradient elution, the constant pressure pumps have a potential problem of gas dissolving at the high pressure end from the gas cylinders used to pressurize the solvent. This introduces gas bubbles into the detector cell which leads to peak tailing. Constant displacement pumps that deliver constant flow rates are therefore used to decrease the adverse effects of changes in flow rates during analysis.
The pressure gauge monitors the pump pressure through the column and indicates any problems such as plugging or leaks, while the sample injector device, usually a loop, is used to introduce the sample into the column.

The column consists of a tube of stainless steel which has been tightly packed with small particles of the material used to effect separation. They are normally short and straight to avoid excessive pressure drops and air pockets which will lead to tailing of peaks. The column is the core of any chromatography and the primary aim in designing any chromatographic procedure must ensure that the full potential of the column is realised in the recorder trace. Both the type of packing put into the column and how well the column is packed are of practical importance.

The widely used column packing materials are rigid solids based on silica matrix which can withstand high pressure. Two different types of particles are available, porous and pellicular. The pellicular particles are made from special glass-beads which are then bonded with the stationary phase. The porous particles can either be spherical or irregular. These packing materials are available in different particle sizes ranging from 50μm down to 3μm in diameter. Large irregular shaped porous particles of about 50μm in
diameter have long separation times and low efficiency due to slow mass transfer in deep bores and large inter-particle channels.

The use of small particles (3-10 μm diameter) result in high column efficiency, though they experience high pressures due to the closely packed particles. The use of small particle size sets a compromises between efficiency, pressure drop, analysis time and reproducibility of packing. Presently most modern high performance liquid chromatography is performed either on pellicular or microporous particle packing.

In this study, a reversed phase column packed with pellicular ODS-material of approximately 10 μm diameter was used.

The most popular detectors in HPLC are the optical detectors. Optical detectors include the UV-vis absorbance, refractive index and recently developed fluorescence detectors. Other type of detectors such as electrochemical, flame ionization, atomic absorption, mass spectrometer, infrared and vidicon-based optical detectors are in an early stage of development and may find increased use in future. The type of detector to be used for a particular problem depends on the characteristics of the solute, the sensitivity required and the convenience and versatility desired.
The detectors used in HPLC can be characterised in various ways. Bulk property or general detectors are those which measure change in some overall physical property of the mobile phase plus the solute, such as the refractive index detector. While solute property or selective detectors are sensitive only to some property of the solute such as UV absorption detectors. Detectors can also be characterised as being destructive when at least part of the sample is altered by the detector itself (such as electrochemical and mass spectrometric detector), or non-destructive such as the commonly used optical detectors that allow sample collection for further quantitative characterization.

UV-Vis absorption detectors constitute the majority of HPLC detectors. This is because they have a wide range of application, are compatible with gradient elution, are non-destructive, and are easy to operate and maintain. They may be of fixed wavelength (UV absorption with detection at 254 or 280nm, or both), or variable wavelength (UV or UV-vis absorption). The advantage of variable wavelength UV-vis absorption detectors is that sensitivity and selectivity can be enhanced by the use of different wavelengths. A change in wavelength may eliminate sensitivity of some compounds while maintaining the sensitivity of others.
The eluted stream in HPLC passes through a flow cell through which transmittance of the monochromatic light is recorded. The sample concentration in the flow cell is related to the fraction of light transmitted through the cell by Beer's law:

\[ \log \frac{I_0}{I} = \varepsilon bc \]

where \( I_0 \) is the incident light intensity, \( I \) is the intensity of the transmitted light, \( \varepsilon \) is the molar absorptivity (molar extinction coefficient) of the sample, \( b \) is the cell pathlength in cm, and \( c \) is the concentration of the sample in moles per litre.

The light absorption HPLC detectors are designed to provide an output in absorbance that is linearly proportional to the concentration of the sample in the cell:

\[ A = \log \frac{I_0}{I} = \varepsilon bc \]

2.2.3. **HPLC Analysis of Vitamins**

HPLC has emerged in recent years as an effective analytical tool in the determination of nutrient and compositional characteristics of foods. It is considered to be the quickest, simplest, most reproducible and least subject to interference, and
therefore a suitable method for analysing complex samples such as food extracts that contain many substances including vitamins. This is perhaps due to the fact that HPLC has two chromatographic phases that enable various compounds to be resolved easily. By adjusting the mobile phase for example, the various forms and isomers of tocopherol as well as carotene can be separated and removed from other compounds on a reversed phase column within a very short time and without affecting the isomers.

The development of a suitable analytical method requires an understanding of the composition of the sample material, particularly the different vitamin forms present and their approximate concentrations. Various HPLC methods have been reported for the determination of both water-soluble and fat-soluble vitamins. These methods may employ either normal phase or reversed phase column and may use varying chromatographic conditions like means of detection and mobile phase composition. The analysis can be conducted on an individual vitamin or number of them simultaneously.

2.2.3.1. Determination of Thiamine and Riboflavin

The determination by high-performance liquid chromatography of water-soluble vitamins including thiamine and riboflavin, singly as well as simultaneously has been known for about fifteen years.
Much of the work on the determination of thiamine and riboflavin in foods by HPLC, however, has been on fortified and enriched foodstuffs, especially when the method of detection was UV absorbance. Determination of thiamine and riboflavin in non-fortified or natural foods has mainly employed fluorescence detection. Since natural foods contain very low levels of thiamine and riboflavin, fluorescence detection is thus preferred because it is more specific and sensitive.

The first liquid chromatographic procedure for the determination of thiamine and riboflavin in natural food system was reported by Van de Weerdhof et al. They used columns of different lengths filled with 20-30μm silica gel and eluted thiamine with 0.1M phosphate buffer solution (PH 6.8) in 10% ethanol, and riboflavin with 0.1M acetate buffer (PH 4.8). Detection was by means of a fluorimetric detector and after thiamine had been oxidized to thiochrome in a post column system. The major weakness in this method was the fact that the column life was reported to be only a few days.

When a method developed by Toma et al. for the determination of thiamine, riboflavin and niacin in rice and rice products failed to work with meat and poultry samples because of the low sensitivity associated with UV detection (used 254nm for detection), and the presence of an array of overlapping peaks, Ang and Moseley developed a more sensitive
method. In this method, riboflavin was converted to lumiflavin by UV irradiation and thiamine to thiochrome by treatment with $K_3Fe(CN)_6$. The vitamins were monitored separately by a fluorescence detector. However, this method requires separate derivatization of the vitamins and does not permit simultaneous determination.

In an effort to shorten this rather lengthy procedure, Kamman et al.\textsuperscript{53} developed a method for simultaneous determination of thiamine and riboflavin. Separation was, however, successful only with fortified food systems of cereal products. The results compared quite well with those obtained by a semiautomatic modification of the AOAC method.

A method was developed by Wimalasiri et al.\textsuperscript{56} for the simultaneous determination of thiamine and riboflavin in foods. The method used a reversed phase column with a mobile phase of methanol-water (40:60) mixture containing 0.005M Pic B$_6$ for separation and fluorescence detection. The study showed that use of UV detector did not resolve riboflavin and thiamine from interfering compounds as did the fluorescence detector. However, simultaneous monitoring of the column effluents required use of two fluorescence detectors both with 360nm excitation filters but one with a 500nm emission filter for detection of riboflavin and the other with a 425nm emission filter for the detection of thiamine. A post column oxidation of thiamine method
was used. In this method also, the samples required purification with a sep-pak cartridge to obtain satisfactory resolution of thiamine and riboflavin.

The method by Wimalasiri et al. was used by Wills et al. so as to compare its results in a wide range of foods with those of the AOAC fluorimetric methods. The values obtained for thiamine by HPLC method were consistently 10% higher than those of the AOAC method while those of riboflavin compared well in both methods.

The other workers who have reported the simultaneous determination of thiamine and riboflavin are Skurry and Fellman et al. The two procedures employ reversed phase systems and fluorescence detection. Skurray's analysis employs a highly alkaline extract that is detrimental to the column life and stability of riboflavin. The method by Fellman et al. employs separate wavelength system for riboflavin analysis because of the presence of interfering substances. The other procedures for the determination of thiamine and riboflavin individually by HPLC have been reported by Botticher et al. and Lumley et al. respectively. However the procedures use fluorescence detectors to monitor the column eluents.

2.2.3.2. Determination of \( \beta \)-Carotene and \( \alpha \)-Tocopherol

Although the determination by HPLC of fat-soluble vitamins including \( \beta \)-carotene and \( \alpha \)-tocopherol in food
systems has been known for well over a decade, the major breakthrough in the analysis of vitamins A and E, however, has been on fortified food systems. β-Carotene and α-tocopherol, as the major forms of vitamins A and E respectively in natural plant food systems, have recently been determined either singly or with other forms for total vitamin activity.

Van de Weerdhof et al. were among the first workers to develop a liquid chromatographic method for determination of vitamin A and β-carotene from natural food systems. They used a column (50 x 0.3 cm i.d) packed with AL$_3$O$_3$ (particle size 18-30 μm) plus H$_2$O as an adsorbent and a mobile phase consisting of 3% ethanol in benzene for vitamin A and 1% ethyl acetate in hexane for β-carotene. Detection was by fluorimetry for vitamin A and colorimetry for β-carotene. However the column life was reported to be only a few weeks.

In an effort to develop a procedure for determination of total vitamin A activity in food samples in a single analysis, Stancher and Zonta came up with a method that separated retinol isomers from carotenoids. Separation was achieved by the use of Lichrosorb Si60 (5 μm) prepacked column and an isocratic mobile phase of ketone-hexane (10:90) mixture. However the method could only be used to
determine total carotene since it does not separate the various carotenoids, especially $\alpha$- and $\beta$-carotenes.

Some workers, who have realised the danger of overestimating vitamin A activity, have reported procedures for separation of various carotenoids found in the complexity of natural food systems. Bushway\textsuperscript{14} developed a method that quantified $\alpha$-carotene and $\beta$-carotene in fruits and vegetables. The method also separated other carotenoids. He used a reverse-phased column with a mobile phase of acetonitrile-methanol-tetrahydrofuran (40:50:4) mixture for separation. Similarly, Speek et al.\textsuperscript{24} developed a method that could be used to determine $\beta$-carotene and estimate vitamin A activity in vegetables, when they realized the possibility of overestimation of vitamin A activity when official AOAC methods are used. For separation a reversed phase column and mobile phase consisting of methanol-acetonitrile-chloroform-water (200:250:90:11) mixture were used.

Other works on the determination of vitamin A activity in foods include that by Hsieh and Karel\textsuperscript{6}. They developed a method with a simple modified extraction procedure for the determination of $\alpha$- and $\beta$-carotene in fruits and vegetables. The samples were extracted with portions of acetone-light petroleum by vigorous shaking or vortexing until the sample was colourless and then separated on a reversed phase system.
Procedures for determination of tocopherols (vitamin E) in foods by HPLC method have only recently appeared in the literature. The various forms of vitamin E that occur in nature can be separated using various column packings. Vatassay et al.\textsuperscript{62} have reported a chromatographic separation of the various tocopherols using pellicular packing material as well as microparticulate silica gel. They used several mixtures of mobile phase for each column, varying the percentage of methanol (0.3-0.8%) in hexane for normal phase columns, and water content (2.5-15%) in methanol for reversed phase columns. They reported that a column packed with microparticulate silica gel had superior efficiency and resolving power that gave baseline separation of $\alpha$-, $\beta$-, $\gamma$- and $\delta$-tocopherols than the other columns.

Some of the HPLC methods for the determination of tocopherols and tocotrienols in foods and feeds include those reported by Thompson et al.\textsuperscript{63}, Cort et al.\textsuperscript{48}, Piironen et al.\textsuperscript{8}, Manz et al.\textsuperscript{64} and Cohan et al.\textsuperscript{65}. Thompson et al. reported a method for the determination of tocopherols and tocotrienols in food and tissues. They used a column packed with Lichrosorb Si60 (5\textmu m) and a mobile phase consisting of 5% ethyl ether in moist hexane. Detection was done fluorimetrically. A similar procedure for the determination of vitamin E content in feedstuffs was reported by Cort et al.\textsuperscript{48}. 
2.2.4. Vitamins Analysis in Kenya

The attention on the analysis of vitamins in Kenya has gained importance by both the food researchers and nutritionists. As noted earlier, this attention has been brought about by the problems of vitamins deficiency that are identified in Kenya, and the associated campaign against the deficiency. Various institutions are involved in vitamin analysis, including the University of Nairobi under the Department of Food Science and Technology, Kenya Medical Research Institute (KEMRI) and the Kenya Bureau of Standards (KBS). These institutions rely largely on the AOAC methods for the analysis of vitamins. However, as already noted, these methods experience various problems such as overestimation of the vitamins, especially vitamins A and E activities in natural food systems where various isomers and forms, some with no vitamin activity, exist. The AOAC methods also involve elaborate purification procedures that are tedious, time consuming and not compatible with routine analysis.

When studying carotene content of some green leafy vegetables of Kenya and the effects of dehydration and storage on carotene retention, Gomez determined carotene contents of the vegetables spectrophotometrically at 450nm; a procedure similar to the official AOAC method. Mwajumwa used the same procedure to determine carotene content when she
performed a nutritional evaluation in her survey of production and utilization of fruits and vegetables of Machakos District. Other works reported on the analysis of vitamins in Kenya are those of Oniango\textsuperscript{23} and Imungi and Potter\textsuperscript{33}.

2.2.5. Objectives of the Present Work

There is a growing public awareness of the relationship between dietary intake and human health in Kenya. Such increased awareness include the understanding of the role of vitamins nutritionally and the presence of vitamin deficiency in many parts of Kenya. The awareness has been brought about by the involvement of food researchers and nutritionists in educating the general public and decision makers on the advantage of alleviating vitamin deficiency. The approach on educating the general public has been mainly to encourage people to grow commonly consumed foods such as vegetables and fruits that are major sources of most vitamins. Such an awareness has led to an increased demand for a rapid and accurate method for the assessment of vitamins.

Most analysts believe that AOAC methods for the analysis of vitamins should be supplemented with new procedures that utilize modern liquid chromatography. The AOAC methods are time consuming and are prone to inaccuracies due to oxidation. HPLC has been considered
to be simple, rapid and less affected by interferences. Since it is versatile in its application, its use in Kenya is expanding rapidly.

Vitamins play an important role in nutrition and in curing some diseases. Because of this role and the fact that current methods of analysing vitamins in foods experience procedural problems, there is need for rapid and simple methods for the analysis of vitamins in foods. Such procedures would be beneficial to food scientists, nutritionists, horticulturists and cancer researchers. Therefore the purpose of this study was to develop rapid, accurate and comparatively simple procedures for simultaneous separation and determination of thiamine and riboflavin on one hand and β-carotene and α-tocopherol on the other hand using high-performance liquid chromatography. The focus of the study centred upon the analysis of some of the natural foods, especially indigenous vegetables, that are commonly consumed by most Kenyans.
CHAPTER 3

EXPERIMENTAL

3.1. Apparatus and HPLC Operating Conditions

3.1.1. Glassware

All the glassware used were cleaned with chromic acid followed by a washing detergent. They were then rinsed with distilled water. Before the glassware were used in the analysis of fat-soluble vitamins (α-tocopherol and β-carotene) they were rinsed with methanol.

3.1.2. HPLC Instrument

The high-performance liquid chromatograph used was model 332 (gradient system) with Beckman pumps (model 110, Beckman Instrument Inc.) which were controlled by a microprocessor system (model 420, Altex Scientific Inc.). The sample was introduced into the column through a sample injector (model 210, Beckman Instrument Inc.) equipped with a 20 μl loop. Separation was achieved by a reversed phase column consisting of stainless steel (30cm long x 4.0mm internal diameter) packed with μBondapack C_{18} material (10μm particle size). The column was obtained from Waters Associates.
The column effluents were monitored by ultraviolet (UV) absorption using a variable wavelength (UV-vis) detector (Hitachi, model 100-40 spectrophotometer). The chromatographic peaks were recorded on a Kipp and Zonen BD41 recorder that was also controlled by the microprocessor system.

3.1.3. HPLC Operating Conditions

Twenty microlitres of both the standard solutions and the sample extracts were injected into HPLC system. The sensitivity used was mainly 0.02 absorbance units full scale (AUFS) although sometimes 0.01 AUFS was used in thiamine and riboflavin analysis. The detection wavelength was 297nm for \( \beta \)-carotene and \( \alpha \)-tocopherol, and 265nm for thiamine and riboflavin. The flow rate was 1.0 ml/min while the chart speed was 0.2 cm/min for the two sets of vitamins.

3.2. Chemical Reagents and Solvents

3.2.1 Chemical Reagents

All the analytical reference compounds were purchased from Sigma Chemical Company, United States of America. The compounds and their catalogue numbers were \( \beta \)-carotene, type III (no. C-9875), DL-\( \alpha \)-tocopherol acetate (no. T-3376), riboflavin (no. R-4500) and thiamine hydrochloride (no. T-4625). Ascorbic acid and 1-hexanesulfonic acid sodium salt were also obtained from Sigma Chemical Company. Takadiastase (diastase
Pure methanol was pumped through the column for 20 minutes after completing the day’s analysis for column cleanup and storage.

**Thiamine and Riboflavin**

The mobile phase used for the elution of thiamine and riboflavin was prepared by mixing 0.01M phosphate buffer plus 0.005M 1-hexanesulfonic acid (pH 7.0), methanol and acetonitrile in the ratio 80:12:8. The mixture was filtered through a millipore filter and deaerated for 10 min. by vacuum before use. Elution of the vitamins was done isocratically.

The phosphate buffer solution with an ion-pair was prepared by weighing 1.742g of di-potassium hydrogen orthophosphate into a 1000ml volumetric flask and dissolving in 200ml distilled water. Then 0.941g of 1-hexanesulfonic acid was added and the mixture diluted to the mark with distilled water. The pH of the solution was adjusted to 7.0 with phosphoric acid.

Following completion of the day’s analysis, a 10% methanol in distilled water was pumped through the column for 20 minutes to prevent precipitation of solvent salts and column malfunctions. Then pure methanol was pumped through for final column cleanup and storage.
3.3. Procedure of Analysis

3.3.1. Preparation of Standard Solutions

(i) \( \beta \)-Carotene and \( \alpha \)-Tocopherol Solutions

The stock solutions of \( \beta \)-Carotene and \( \alpha \)-tocopherol acetate, each containing 100\( \mu \)g/ml were prepared by weighing 10mg of the standard reagents into 100ml volumetric flasks. The reagents were dissolved in some distilled n-hexane and then diluted to the mark. The stock solutions, which were kept under refrigeration conditions, could be used for a period of about four weeks.

A working solution consisting of \( \beta \)-carotene and \( \alpha \)-tocopherol mixture was prepared by taking an aliquot from each stock solution (such as 1.0ml) into a 100ml round bottomed flask and treating it according to the extraction procedure given in section 3.3.2(c)(i) below. However, solvent extraction was done once, while the residue after vacuum evaporation was dissolved in 10ml of ethanol. When kept under refrigeration conditions, this solution could be used for about one week.

(ii) Thiamine and Riboflavin Solutions

Stock solutions containing 100\( \mu \)g/ml of thiamine and riboflavin each were prepared by weighing 10mg of each of the reference reagents, that have been dried overnight over \( \text{P}_{2}\text{O}_{5} \) in a desiccator into, 100ml volumetric flask. 20.0ml of 0.1M HCl was added to each
flasks, shaken to dissolve the reagents and then diluted to the mark with distilled water. These stock solutions were kept in the refrigerator and could be used for a period of about three weeks.

A working solution consisting of a mixture of thiamine and riboflavin was prepared by taking an aliquot of 1.0ml from each stock solution into a 10ml volumetric flask and diluted to the mark with distilled water. The working solution was freshly prepared every day of analysis.

3.3.2. Sample Preparation and Extraction

(a) Sampling

Sampling of the foods was carried out during both wet and dry seasons. The samples were obtained from various places in Kenya. Most of the foods such as leafy vegetables, potatoes, tomatoes, green grams and green peas were purchased from vegetable kiosks in Nairobi. These foods were brought from various parts of the country, notably Kiambu, Muranga, Nyeri, Machakos and some parts of the Rift Valley. In the kiosks, the foods were sold in bundles of 0.5 to 1.0kg. Four to six bundles of each food were purchased and pooled for analysis.

Some of the food samples namely *gynandropsis gynandra*, nightshade, bananas, beans and kales were obtained in bundles of one to two kilograms from a Kisii household garden. Similarly, some samples
including Kale, tomatoes, cauliflower leaves, spinach, amaranthus, nightshade and onion (stem and leaves) were obtained from households and kitchen gardens in Nairobi and Ngong area.

The sampled foods were analysed the same day. If analyses could not be performed the same day, the samples were stored under refrigeration conditions in plastic bags for analysis later. However, most foods especially green vegetables were not kept for more than two days before they were analysed. Other foods such as dry legumes could be kept for up to three months before analysis.

(b) Sample Pretreatment

Samples of vegetables, banana, potatoes and onion stem were trimmed to remove fibrous, dried or inedible parts, leaves as well as peelings according to common household practices so that only the edible portions were analysed. The trimmed samples were cut with a knife, homogenized into fine paste by grinding in a mortar and analysed immediately in duplicates. Dry samples such as legumes were ground into a fine powder to pass through a No. 30 Sieve and immediately analysed in duplicates.

(c) Sample Extraction

All extraction steps were performed under subdued light and/or in glass apparatus covered with aluminium
foi. Some steps, especially in the extraction of \( \beta \)-carotene and \( \alpha \)-tocopherol were carried out promptly and fast to avoid vitamin oxidation by air.

(i) Extraction of \( \beta \)-Carotene and \( \alpha \)-Tocopherol

A sample of 2.5 or 5g was weighed and transferred into a 150ml round bottomed flask. 50ml of 0.5M ethanolic potassium hydroxide, 5.0ml of 10% sodium sulphite (\( \text{Na}_2\text{SO}_3 \)) solution (\( \text{W}/\text{v} \)) and 0.3g of ascorbic acid were added. The mixture was dispersed in the solution with a glass rod and refluxed over boiling water (94\(^{\circ}\)C) for 30 min. The mixture was then immediately corked and cooled over tap water to room temperature. 30ml of n-hexane was added to the mixture, shaken thoroughly for two minutes and allowed to separate. The hexane layer was decanted into a 250ml separating funnel and corked. The residue was similarly re-extracted with 30ml n-hexane five times and the n-hexane layer decanted to the separating funnel. The combined n-hexane layer in the separating funnel was washed with 50ml of 1.0M KOH solution followed by portions of 50ml of distilled water until there was no coloration on phenolphthalein solution (usually five portions of distilled water were enough). The hexane layer was then dried by filtering over anhydrous sodium sulphate and evaporated to almost dryness in a rotatory evaporator at 40\(^{\circ}\)C. The residue
was immediately dissolved in 1.0-10.0ml of ethanol (depending on the expected concentration) and an aliquot of the solution was injected into the HPLC system for analysis.

(ii) Extraction of Thiamine and Riboflavin

A portion of 10g of wet samples such as leafy vegetables or 2.5g of dry samples such as legumes was weighed and transferred into a 100ml conical flask. 20ml of 0.26M HCL was added to the flask and the sample dispersed with a stirring rod. The flask was then covered with an aluminium foil and the mixture hydrolysed by heating over boiling water for 45-60 min. The contents were occasionally shaken to prevent lumping.

After cooling the mixture over tap water the pH was adjusted to 4.5 with 2.5M sodium acetate solution. A pH meter (metrolim 632 model) was used to adjust the pH of the mixture. 0.1g of takadiastase and 0.3g of papain were added and thoroughly mixed by stirring. The mixture was then incubated for two and half hours at 45°C, cooled to room temperature over tap water and filtered by vacuum using an ashless filter-paper (Whatman paper No. 42). An aliquot of the filtrate was injected into HPLC for analysis.
Recovery samples were prepared by adding known amounts of the standard reagents to the homogenised sample before extraction. The samples were then extracted and analysed as per above procedures.

(d) Reproducibility Studies

Some food samples (0.1 to 1.0kg each) were pretreated as above to obtain a homogeneous sample. Five subsamples were removed, extracted and analysed using the procedures described above. Coefficients of variation were calculated to determine the reproducibility of the methods.

3.4 Quantification of the Results

Peak identification was done by comparing the retention times of the standard and sample solutions. The standard solution was injected first followed by five injections of sample extract solutions and then the standard solution. Peak height was used for quantification since it was shown to be linear with concentration over the working range. A linear calibration curve for each vitamin was constructed. Quantification was accomplished by comparing the peak heights of the samples to the peak heights of the standards. The concentration of the vitamins was calculated according to the formula given in appendix.
RESULTS AND DISCUSSION

The separation and quantification of vitamins such as thiamine, riboflavin, β-carotene and α-tocopherol from foods of plant origin require careful consideration of the chemical interdependences between the sample matrix and the compounds of interest. Such a relationship dictates the approach adapted to procedures such as extraction and determination. Although many of the fat-soluble and water-soluble vitamins can be separated from a standard mixture the complexity of the food sample and the differing chemical characteristics of vitamins make it difficult to determine the two types of vitamins simultaneously.

The differing chemical characteristics of fat-soluble and water-soluble vitamins hinder the use of a similar extraction procedure for the two groups of vitamins. The development of a suitable analytical method, thus, requires an understanding of the composition of the starting material, including the different vitamin forms present and their approximate concentration. In this study, in addition to the above considerations, the conditions chosen were a compromise between the needs for the separate assays of the two groups of vitamins (β-carotene and α-tocopherol, and
thiamine and riboflavin) and the desire to keep the equipment needed simple. The two groups of vitamins will be discussed separately.

4.1. \( \beta \)-Carotene and \( \alpha \)-Tocopherol

The procedure used for extraction of \( \beta \)-carotene and \( \alpha \)-tocopherol from foods was alkaline hydrolysis followed by solvent extraction to remove unsaponifiable materials. Alkaline hydrolysis as a digestive method helps to break down the plant material and thus liberate the vitamins from the protective coatings. Apart from converting vitamin esters to their corresponding alcohols, alkaline hydrolysis also converts fats to soaps which can be separated from the vitamins by solvent extraction. Similarly, a large number of pigments and other substances that might otherwise interfere in the measurement are broken down into small water-soluble molecules. However, the vitamins, especially \( \alpha \)-tocopherol, are very sensitive to oxidation in the presence of an alkali. To avoid destruction of the vitamins during the saponification step, ascorbic acid and sodium sulphite were added as antioxidants.

An ultraviolet absorbance scan of \( \alpha \)-tocopherol standard showed a broad absorbance range with a maximum at 297nm in the eluant used (Figure 4.01a, p. 93). The spectrum shows that the vitamin has a minimum absorbance at more than 300nm. On the other hand, \( \beta \)-
carotene gave an absorbance maximum at 450nm with minimum absorbance at less than 400nm (Figure 4.01b, p. 93). However, α-tocopherol has very low absorption coefficient even at maximum absorbance \(^{16,38}\), while β-carotene has high absorption coefficient \(^{24}\). β-Carotene was found to absorb significantly at 297nm thus enabling the simultaneous detection of β-carotene and α-tocopherol to be monitored at 297nm.

Figure 4.02 (p. 94) shows the chromatogram obtained from the separation of a standard mixture under the chromatographic conditions used in this study. The development of a chromatographic system with sufficient resolution and flexibility to quantify the vitamins from organic extracts of the food samples involved the examination of various solvent systems. The solvent systems containing combinations of methanol, acetonitrile, chloroform and water were examined for the separation suitability on a reversed phase column. The variation of the system was to provide the degree of separation required within practical analytical time.

A mobile phase consisting of a mixture of methanol-acetonitrile (60:40) first used on the studies with standards showed that α-tocopherol and β-carotene eluted from the column after 5 min and 10 min respectively as sharp peaks. The separation achieved with the vitamin standards indicated that quantification could be readily accomplished. These
conditions were extended to food extracts where it was found to work on samples that contain very low amounts, or none of the other forms of carotene. For some samples such as carrots that contain high amounts of \( \alpha \)-carotene\(^6,14 \) the solvent system consisting of methanol-acetonitrile (60:40) does not enable the separation of \( \alpha \)- and \( \beta \)-carotenes.

The reason for selecting the mobile phase conditions utilized in this study is demonstrated in Figure 4.03 (p. 95). The figure depicts the necessity of adding some amount of water to the mobile phase for the separation of \( \alpha \)- and \( \beta \)-carotenes in the carrot extract. The partition of \( \alpha \)- and \( \beta \)-carotenes between the stationary and the mobile phase can be influenced by varying the water content in the mobile phase. The addition of a small amount of water (4-10%) in the methanol-acetonitrile mixture enables separation to be achieved.

The solubility of these compounds in the mobile phase, however, decreases with increasing water content, resulting in longer retention times. The addition of 10% water into the mobile phase, for instance, increases the retention time of \( \beta \)-carotene to over 30 min. Thus the retention time of the carotenes was controlled by the addition of some amount of chloroform to the mobile phase, such as used in this study.
study. Figure 4.04 to 4.06 (pp. 96 to 98) show typical chromatograms for some food extracts analysed by this technique.

The calibration curves for \( \beta \)-carotene and \( \alpha \)-tocopherol are shown in Figure 4.07 (p. 99). The peak heights were directly proportional to the concentration in the range examined. Absorbance of vitamins, expressed as peak height and the concentration of the vitamins injected conformed to Beers's law up to 30 \( \mu \)g/ml for \( \beta \)-carotene and 50 \( \mu \)g/ml for \( \alpha \)-tocopherol. The statistical analysis gave correlation coefficients of 0.9970 for \( \beta \)-carotene and 0.9992 for \( \alpha \)-tocopherol.

The detection limits, obtained as detector response that was double the response of the detector noise, were 2.0 \( \mu \)g/ml for \( \alpha \)-tocopherol and 1.0 \( \mu \)g/ml for \( \beta \)-carotene. The foods analysed had a wide range of vitamin contents, ranging from about 0.05 to over 10.0mg per 100g of food. The size of the sample used for extraction and the final volume used to dissolve the extract residue after vacuum evaporation were chosen such that the concentrations could fall within the straight line curve. Food samples that had more than 1.0mg of \( \beta \)-carotene and \( \alpha \)-tocopherol per 100g required a sample size of 2.5g. The residue, after vacuum evaporation, was dissolved in between 1.0 and 10.0ml of ethanol depending on the expected concentration. For example, the analysis of amaranthus
required a sample size of 2.5g for extraction and the residue was dissolved in 10ml of ethanol (Figure 4.04, p. 96).

Samples with much lower concentrations of vitamins required 10.0 or 20.0g sample size for extraction. The dissolution volume of the residue after vacuum evaporation was 1.0 or 2.0ml. The analysis of ripe banana, for instance, required 20.0g of the sample for extraction and the residue was dissolved in 1.0ml (Figure 4.04, p. 96). Samples that had higher concentrations of β-carotene but low values of α-tocopherol, for example carrots, required higher sample size and lower dissolution volumes in order to be able to quantify α-tocopherol. Then the extract would be diluted for the quantification of β-carotene.

The recovery data shown in Table 4.1 (p. 84) were obtained by adding known amounts of β-carotene and α-tocopherol standards to the homogenized food samples that were ready for extraction, and comparing the increased calculated vitamins content to the amounts added. The same homogenized sample was analysed for the vitamin content to enable comparison to be made. The amount of pure vitamins added was comparable to the expected amount of vitamins in the quantity of the food used for extraction. Thus the peak heights of recovery samples were approximately twice those of the original samples (Figure 4.08, p. 100).
The recovery study was necessary as a means of examining the extent of destruction of the vitamins during the extraction procedure. The vitamins are labile in the presence of oxygen and other oxidizing agents. The obtained recovery range was between 89.3 to 99.2% for $\beta$-carotene and 88.1 to 98.8% for $\alpha$-tocopherol. The mean recoveries were 95.0% for $\beta$-carotene and 94.7% for $\alpha$-tocopherol. No significant difference was seen for recoveries of $\beta$-carotene and $\alpha$-tocopherol from the samples studied. As evidenced by the recovery data the vitamins appear not to be affected significantly by oxidation, especially during saponification and extraction stages.

For determining the reproducibility of this method and also the adequacy of the grinding procedure, some food samples were analysed five times each for their $\beta$-carotene and $\alpha$-tocopherol content. The results are given in Table 4.2 (p. 85). The coefficients of variation ranged from 2.0 to 7.3% for $\beta$-carotene and 2.1 and 6.3% for $\alpha$-tocopherol, with most of them below 5.0%. The results indicate that the method is reproducible and that sample homogeneity is not a problem with the grinding technique.

The method developed in this study was used to determine $\beta$-carotene and $\alpha$-tocopherol in some vegetables, legumes and fruits that are commonly consumed in Kenya. The foods assayed for the vitamins content are listed in Table 4.3 (p. 86) and are
identified by their local as well as botanical names. The values obtained are shown in Tables 4.4a and 4.4b (pp. 87 and 88).

Comparing Tables 4.4a and 4.4b (pp. 87 and 88), it is very clear that green vegetables, especially leafy vegetables are the best sources of both β-carotene and α-tocopherol. Other foods contain relatively lower values of these vitamins, except carrots that contain high value of β-carotene. The indigenous vegetables such as amaranthus, nightshade, pumpkin leaf, gynandropsis gynandra and unconventional vegetables such as bean leaf (though consumed in some parts of Kenya) are superior sources of both β-carotene and α-tocopherol in comparison to exotic vegetables such as cabbage and lettuce. In the ranking of the vegetables found in Machakos in their micronutrient importance, Mwajumwa arranged them in terms of β-carotene content as bean leaves, nightshade, gynandropsis gynandra, pumpkin leaves and amaranthus (all types) in that order. It was also noticed that some vegetable parts which are not consumed such as cauliflower leaf or less consumed such as onion leaf contain higher contents of β-carotene and α-tocopherol than the parts consumed (cauliflower curd and onion stem respectively).

The data given in Table 4.4a (p. 87) show that there is some relationship between the content of β-carotene and α-tocopherol in vegetables. Such a
relationship is not clearly noticeable in other foods (Table 4.4b, p. 88) as some foods, especially legumes contain other forms of vitamin E in large quantities. From the results obtained for vegetables, the sample with a high content of \( \beta \)-carotene is likely to have a high content of \( \alpha \)-tocopherol and vice versa. However, exceptions to this relationship are bean leaf and \( G. \ gyanadra \) where the \( \alpha \)-tocopherol contents are lower than half the contents of \( \beta \)-carotene. The linear correlation analysis of the data gave a correlation coefficient of 0.680, signifying the existence of some form of a relationship. This comparison was noted without further comment as more study is needed.

The values of \( \beta \)-carotene and \( \alpha \)-tocopherol obtained for various types of foods are generally within the range of the values reported elsewhere\(^5, 16, 18, 24, 25, 68\). While some of the \( \alpha \)-tocopherol values obtained in the study are comparable to those cited in the literature\(^5, 16, 68\) especially for tomato, banana, beans, peas and carrots, the \( \alpha \)-tocopherol values of most foods show a wide variation in the literature. This may be accounted for by the variation in species and growing conditions\(^8\).

Some \( \beta \)-carotene values obtained in the study were within the ranges reported by Gomez\(^{25}\) and Mwajumwa\(^{18}\), especially with respect to leafy vegetables. However, some values obtained, for \( sukuma wiki \), nightshade, \( gynandropsis gynandra \) and cabbage, for example, are
slightly lower than those reported by Gomez and Mwajumwa. Some of the reasons that may be advanced for these differences include the fact that the method used by these workers does not separate various forms of carotene such as $\alpha$- and $\beta$-carotenes, and many other carotenoids. The method they used does not separate $\beta$-carotene from the less active $\alpha$- and $\gamma$-carotene as these compounds have a polarity similar to that of $\beta$-carotene. These forms of carotene and their isomers are found in most fruits and vegetables.

A rapid destruction of carotenes occurs when plant cells are ruptured, presumably enzymatic in nature. Since most of the samples analysed were purchased from vegetable vending kiosks or brought from Kisii this effect would be expected. These samples had stayed for more than a day from the time they were harvested and since they were kept under normal conditions, enzymatic destruction of carotene was rapid. This was unlike those samples analysed by Gomez.

Another reason why the values obtained cannot be directly compared with those published is the fact that the levels of $\beta$-carotene in many of the fruits and vegetables are dependent on the season, variety and maturity of tissues.

The method was extended to the determination of $\alpha$-tocopherol and $\beta$-carotene in some seed oils. The results are shown in Table 4.4c (p. 89). The seed oils
hardly contain \( \beta \)-carotene except in a few cases (Neem seed, Orange seed, Safflower and Erthrina Abyssinica seed) where it was recorded in trace amounts. But the seed oils were found to contain \( \alpha \)-tocopherol in varying concentrations. Safflower, covo and corn oils were found to contain the highest amounts of \( \alpha \)-tocopherol. The value obtained for safflower is comparable to that reported by Deluca\(^6\). Other seed oils seem to contain high contents of other forms of vitamin E such as \( \beta \)-tocopherol and \( \alpha \)-tocotrienol. This can be confirmed from the large peaks that appear in the chromatograms around the region where \( \alpha \)-tocopherol appear (Figure 4.06, p. 98). Figure 4.06 (p. 98) also shows that legumes contain large amount of other forms of vitamin E.

The extraction procedure used in this study gave some problems when it was applied to the oils. During the washing stage, some seed oils gave a thick soapy materials that affected the separation of the organic and aqueous layers. In such cases, the oils were diluted in hexane or the mobile phase and analysed thereafter\(^6\).

4.2. Thiamine and Riboflavin

In nature thiamine and riboflavin occur bound to protein in free form or in ester forms. In plant foods, the free form of thiamine dominates while the phosphates forms of riboflavin, especially flavin
ademine dinucleotide (FAD), dominates. During the extraction procedure a mild acid hydrolysis is used to release the protein bound vitamins. Since the vitamins especially thiamine, are unstable in alkaline or neutral conditions, the acid digestion should be distinctly acidic (pH < 3.0). Most of the food materials are substantially basic, such that the use of 0.1M HCl as recommended by most workers was found inadequate. Thus an acid solution with a higher concentration (0.26M) was used. Even with this, some samples were so basic that they required pH adjustment before digestion.

The phosphate esters of thiamine and riboflavin were converted into the free forms by enzymatic treatment. An enzyme, takadiastase, was used because of its wide enzymatic activities. The diastase content of the enzyme mixture has the additional function of hydrolysing starch. Since starch and proteins interfere with extraction, the hydrolysis of starch enables the removal of much of the solid material and permits ready filtration.

The extraction technique developed in this study resulted in a greater vitamin concentration in the supernatant aliquot than that which results from the normal approved methods. The minimum possible volume of the acid solution was used during hydrolysis. A volume equal in millilitres to eight times or less the weight of the dry sample was used instead of a volume equal in
millilitres to ten or more. Samples containing high percentages of moisture contents (such as leafy vegetables) required volumes equal in millilitres to twice the wet weight of the sample in grams. This increased the concentration, and allowed detection of the vitamin by ultraviolet (UV) absorbance. Vitamins concentration resulting from approved methods of extraction are below the limits of UV detection for most samples.

The ultraviolet absorbance scan of thiamine and riboflavin standards showed that thiamine has two major absorbance maxima at 265 and 232nm (Figure 4.09, p. 101). Riboflavin has two sharp absorbance maxima at 256 and 214nm (Figure 4.10, p. 102). Since riboflavin has higher absorbance ratio as compared to thiamine, a wavelength of 265nm which is one of the maxima absorbance of thiamine and near the absorbance maximum of riboflavin was chosen for detection. The use of UV absorbance for detection enabled the detection of thiamine without converting it to thiochrome, and permitted simultaneous measurement with riboflavin; thereby simplifying the entire procedure.

The mobile phase selected for use in this study was the result of the examination of elution profiles of the vitamins as affected by the organic solvent concentration, buffer concentration and the use of ion-pair. Thiamine is strongly ionic while riboflavin is nonionic. According to these properties, the separation
of the vitamins on a reversed phase column would be totally dependent on the polarity of the mobile phase. The amount of the organic solvent (methanol and acetonitrile) in the buffer solution was found to affect the retention time of riboflavin, and to a lesser extent thiamine. High organic solvent content (>25%) makes riboflavin to have very low retention time such that its peak does not separate from thiamine. The studies with food extract using a mobile phase containing more than 25% organic solvent showed that the vitamins were eluting in an area covered by a large array of unidentified peaks (at the retention times of between 2 and 8 min).

On reducing the concentration of the organic solvent in the mobile phase to less than 10% the retention time of riboflavin increased to an extent that the peak became too broad to quantify effectively (>30 min). The retention time of thiamine only increased slightly but without separating from the unidentified peaks. The amount of organic solvent chosen (20%) enabled riboflavin to elute just after the large block of unidentified peaks, as shown in Figure 4.11 (p.103). However, under these conditions thiamine eluted from the column with other unidentified substances.

The buffer concentration was found to affect the retention time of thiamine because of its ionic nature. The use of the buffer concentration greater than 0.01M
makes thiamine to elute within the area covered by the large unidentified peaks. Use of a buffer of concentration less than 0.01M (for example 0.005M) increased the thiamine retention time but the increase was small as the peak could not separate out from the unidentified peaks. In introducing an organic counter ion, such as 1-hexanesulfonic acid, the cationic thiamine forms an ion-pair in the mobile phase and the ion-paired complex partitions with the stationary phase. The system is often described as a dynamic ion exchange column. The net effect of the ion-pair partitioning is that the elution time of thiamine is increased. Thus the use of 1-hexanesulfonic acid increased the retention time of thiamine such that it eluted after riboflavin, where it was well away from the large array of unidentified peaks. The adjustment of the amount of the organic solvent in the mobile phase results in the alteration of the primary equilibrium of the system. Changing of other factors such as the pH or the use of an ion-pair affects the secondary equilibrium. Hence both the primary and secondary equilibria were adjusted for the control of selectivity and retention of the vitamins so that the peaks were free from background interferences and that the vitamins were eluted at convenient minimum time. Figure 4.12 (p.104) shows the separation of a mixture of thiamine and riboflavin standards using the
solvent phase selected and utilized in this study. Figure 4.13 (p. 105) presents typical chromatograms obtained for some of the food extracts assayed by this technique.

Figure 4.14 (p.106) shows the calibration curves for thiamine and riboflavin. The linear correlation analysis gave the correlation coefficients of 0.99918 for thiamine and 0.99870 for riboflavin. The detection limits were 1.0 pg/ml for thiamine and 0.5 μg/ml for riboflavin. Thiamine is unstable to heat while riboflavin is easily destroyed by light, especially ultraviolet light. In order to assess the extent of destruction of the vitamins during extraction, recovery experiments were done on some food samples. The procedure used for recovery studies is similar to that given in section 4.1 above on the recovery of β-carotene and α-tocopherol. Table 4.5 (p. 90) gives the results obtained. The recoveries ranged from 88.9 to 97.5% for thiamine and 89.0 to 97.0% for riboflavin. The average recoveries were 92.0% for thiamine 93.7% for riboflavin. This recovery data show that there are very low losses of vitamins during extraction.

The reproducibility of the method and the adequacy of the grinding procedure were studied by analysing some food samples five times each for their thiamine and riboflavin contents. The results obtained are shown in Table 4.6 (p. 91). The coefficients of
variation obtained ranged from 4.4 to 9.0% for thiamine and 3.1 to 5.6% for riboflavin. The results indicated that homogeneity was attained by the grinding procedure and that the method was reproducible.

The method developed in this study was used to determine thiamine and riboflavin contents in foods. The results obtained are given in Table 4.7 (p. 92). It is very clear from the results that green vegetables such as spinach, kales, nightshade, cowpea leaves and amaranthus are good sources of riboflavin but poor sources of thiamine. The thiamine levels in the green vegetables are so low that they cannot be detected by this method. Some foods such as tomato, onion stem and banana (unripe) contain very low contents of both thiamine and riboflavin. Riboflavin was recorded in trace amounts or not detected at all, while thiamine would not be detected. Legumes such as green peas, pigeon peas, beans and green grams on the other hand are good sources of thiamine and to some extent riboflavin.

The levels of thiamine and riboflavin found in the foods analysed were consistent with the values reported\textsuperscript{4,68,69}. Riboflavin levels for some vegetables such as nightshade, kales and cowpea leaves were however, slightly higher than those reported\textsuperscript{69}. An explanation for this slight variation in the levels may be due to the fact that in the use of the AOAC methods
recoveries are low\textsuperscript{17}. Such loses may come during the purification stage where the vitamin is exposed to light.

When the method was applied to some fortified commercial foods, the values obtained compared well with the values on their labels. The values obtained for Bournvita, for example, compared well with those on the label; 1.90mg per 100g compared with 2.0mg per 100g and 2.55mg per 100g compared with 2.70mg per 100g for thiamine and riboflavin respectively.

4.3. Conclusions and Recommendations for Further Work

The study has presented fast, simple, accurate and reproducible methods for simultaneous determination of β-carotene and α-tocopherol on one hand, and thiamine and riboflavin on the other in some commonly consumed foods in Kenya. The HPLC methods have distinct advantages over the other methods discussed in this study. HPLC methods are much more simplified in a number of ways, such as it eliminates the lengthy purification steps, allows simultaneous determination, and follows simple procedures. Thus the methods would be used as routine procedures by many workers including nutritionists and horticulturalists. Similar HPLC studies can be extended to the analysis of other vitamins in foods.
The simultaneous determination of β-carotene and α-tocopherol uses a selected mobile phase that provides a chromatographic separation of various forms of carotene as well as tocopherol. Similarly, a modified extraction technique which concentrates the vitamins to within range for UV detection, in combination with an appropriate mobile phase resulted in rapid and simultaneous determination of thiamine and riboflavin in foods. The method affords greater handling simplification as compared to the approved manual techniques by eliminating both the conversion to thiachrome and the need to correct for background fluorescence.

The simultaneous determination procedures results in substantial reduction in the analysis time. Some workers who have compared the HPLC with official AOAC methods have reported that HPLC methods are faster\(^{56-59}\). Wimalasiri et al.\(^{56}\), for example, estimated that the reduction in the analysis time by the simultaneous HPLC procedure amounts to one-third of the total time taken in the separate analysis by the AOAC methods.

The recovery data suggest that lower losses of the vitamins occurred in the procedures. This has been confirmed by some workers who have compared HPLC and AOAC methods\(^{47}\). It is, however, recognized that recovery studies only test the efficiency of the recovery of pure compounds and therefore do not measure the efficiency of extraction from the food.
The reproducibility studies show that optimum homogeneity of the sample was achieved by the grinding method used. Similar results were obtained by Bushway\textsuperscript{14}.

From the results obtained in the analysis of the vitamin contents in some foods (Tables 4.4a, 4.4b, 4.7, pp. 87, 88, 92) it is apparent that green vegetables are good sources of $\beta$-carotene, $\alpha$-tocopherol and to some extent, riboflavin. This is more so with indigenous vegetables, some of which grow wildy such as amaranthus. The results would justify the call for increased consumption of indigenous vegetables. The high values of both $\beta$-carotene probably with and $\alpha$-tocopherol in indigenous vegetables will help in the prevention of night blindness among other deficiency diseases.

Further analysis of the vitamins in more indigenous vegetables, some of which are less consumed such as Irish potato leaves and bush Okra (Chorchorus Olitorius) is, however, required. Similarly, analysis of other main vitamins in indigenous vegetables as well as other foods will yield useful data to support encouragement of production and consumption of these foods. Such studies will form the basis in the campaign against vitamin deficiency problems in Kenya.
Table 4.1 Recovery of β-carotene and α-tocopherol added to some foods

<table>
<thead>
<tr>
<th>Food</th>
<th>Amount (μg)</th>
<th>Percentage</th>
<th>Amount (μg)</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Spinach</td>
<td>80.0</td>
<td>76.5</td>
<td>100.0</td>
<td>96.8</td>
</tr>
<tr>
<td>2. Kale (Sukuma Wiki)</td>
<td>50.0</td>
<td>48.4</td>
<td>80.0</td>
<td>78.2</td>
</tr>
<tr>
<td>3. Nightshade</td>
<td>50.0</td>
<td>49.4</td>
<td>80.0</td>
<td>79.0</td>
</tr>
<tr>
<td>4. Bean leaf</td>
<td>50.0</td>
<td>49.6</td>
<td>80.0</td>
<td>77.2</td>
</tr>
<tr>
<td>5. Amaranthus (LL)</td>
<td>50.0</td>
<td>47.8</td>
<td>60.0</td>
<td>54.3</td>
</tr>
<tr>
<td>6. Onion leaf</td>
<td>70.0</td>
<td>64.9</td>
<td>100.0</td>
<td>89.5</td>
</tr>
<tr>
<td>7. Cowpea leaf</td>
<td>60.0</td>
<td>54.7</td>
<td>60.0</td>
<td>55.2</td>
</tr>
<tr>
<td>8. Potato</td>
<td>80.0</td>
<td>71.4</td>
<td>100.0</td>
<td>98.6</td>
</tr>
<tr>
<td>9. Tomato</td>
<td>80.0</td>
<td>74.8</td>
<td>80.0</td>
<td>70.5</td>
</tr>
<tr>
<td>10. Banana (Unripe)</td>
<td>100.0</td>
<td>97.4</td>
<td>100.0</td>
<td>93.4</td>
</tr>
</tbody>
</table>

LL - Long Leafed
Table 4.2 Reproducibility results of \( \beta \)-carotene and \( \alpha \)-tocopherol in some foods

<table>
<thead>
<tr>
<th>Food</th>
<th>Mean content (( \mu g/100g ))</th>
<th>Coefficient of variation (%)</th>
<th>Mean content (( \mu g/100g ))</th>
<th>Coefficient of variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kale (Sukuma Wiki)</td>
<td>4937 ( \pm ) 226.8</td>
<td>4.6</td>
<td>4390 ( \pm ) 140.2</td>
<td>3.2</td>
</tr>
<tr>
<td>Spinach</td>
<td>3150 ( \pm ) 132.9</td>
<td>4.2</td>
<td>4270 ( \pm ) 125.2</td>
<td>2.9</td>
</tr>
<tr>
<td>Amaranthus (LL)</td>
<td>7652 ( \pm ) 236.2</td>
<td>3.1</td>
<td>7130 ( \pm ) 222.1</td>
<td>3.1</td>
</tr>
<tr>
<td>Pumpkin leaf</td>
<td>8584 ( \pm ) 269.6</td>
<td>3.1</td>
<td>7540 ( \pm ) 174.2</td>
<td>2.3</td>
</tr>
<tr>
<td>Cowpea leaf</td>
<td>7590 ( \pm ) 220.0</td>
<td>2.9</td>
<td>6455 ( \pm ) 197.4</td>
<td>3.1</td>
</tr>
<tr>
<td>Nightshade</td>
<td>6758 ( \pm ) 177.6</td>
<td>2.6</td>
<td>7280 ( \pm ) 151.2</td>
<td>2.1</td>
</tr>
<tr>
<td>G. gyandra</td>
<td>7585 ( \pm ) 149.9</td>
<td>2.0</td>
<td>3035 ( \pm ) 112.4</td>
<td>3.7</td>
</tr>
<tr>
<td>Lettuce</td>
<td>48 ( \pm ) 3.5</td>
<td>7.3</td>
<td>235 ( \pm ) 14.7</td>
<td>6.3</td>
</tr>
<tr>
<td>Banana (Unripe)</td>
<td>151 ( \pm ) 6.8</td>
<td>4.5</td>
<td>230 ( \pm ) 10.8</td>
<td>4.6</td>
</tr>
<tr>
<td>Green Peas</td>
<td>202 ( \pm ) 12.1</td>
<td>6.0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\( a \) Mean \( \pm \) SD (\( N=5 \))
Table 4.3 Some foods assayed for the vitamin contents

<table>
<thead>
<tr>
<th>Common names</th>
<th>Local names</th>
<th>Botanical names</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Kale</td>
<td>Sukuma wiki (Kiswahili)</td>
<td>Brassica oleracea, var. acephala</td>
</tr>
<tr>
<td>2. Nightshade</td>
<td>Managu (Kikuyu)</td>
<td>Solanum nigrum</td>
</tr>
<tr>
<td>3. Spinach</td>
<td>-</td>
<td>Spinacea oleracea</td>
</tr>
<tr>
<td>4. Cabbage</td>
<td>-</td>
<td>Brassica oleracea, var. capitata</td>
</tr>
<tr>
<td>5. Cowpea leaf</td>
<td>Kunde (Kiswahili)</td>
<td>Vigna unguiculata</td>
</tr>
<tr>
<td>6. Bean leaf</td>
<td>Rikuneni (Kisii)</td>
<td>Pheseolus vulgaris</td>
</tr>
<tr>
<td>7. Bean seed</td>
<td>Chingende (Kisii)</td>
<td>&quot;</td>
</tr>
<tr>
<td>8. Pumpkin leaf</td>
<td>Marenge (Kikuyu)</td>
<td>Cucurbita pepo</td>
</tr>
<tr>
<td>9. Amaranthus</td>
<td>Terere (Kikuyu)</td>
<td>Amaranthus hybridus (LL)</td>
</tr>
<tr>
<td>10. Cauliflower</td>
<td>Emboga (Kisii)</td>
<td>Amaranthus lividus (SL)</td>
</tr>
<tr>
<td>11. -</td>
<td>Chinsaga (Kisii)</td>
<td>Brassica oleracea, var. botrytis</td>
</tr>
<tr>
<td>12. Lettuce</td>
<td>-</td>
<td>Gynandropsis gynandra</td>
</tr>
<tr>
<td>13. Onion</td>
<td>Egetungwuo (Kisii)</td>
<td>Lactuca sativa</td>
</tr>
<tr>
<td>14. Carrots</td>
<td>-</td>
<td>Allium fistulosum</td>
</tr>
<tr>
<td>15. Tomatoes</td>
<td>Chinyanya (Kisii)</td>
<td>Allium fistulosum</td>
</tr>
<tr>
<td>16. Potatoes (Irish)</td>
<td>Waru (Kikuyu)</td>
<td>Duncus carota</td>
</tr>
<tr>
<td>17. Bananas</td>
<td>Amatoke (Kisii)</td>
<td>Lycopersicon esculentum</td>
</tr>
<tr>
<td>18. Pigeon peas</td>
<td>-</td>
<td>Solanum tuberosum</td>
</tr>
<tr>
<td>19. Green grams</td>
<td>Ndengu (Kiswahili)</td>
<td>&quot;</td>
</tr>
<tr>
<td>20. Green peas</td>
<td>-</td>
<td>Cojanus cajan</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pisum sativum</td>
</tr>
</tbody>
</table>

86
Table 4.4a Average contents of β-carotene and α-tocopherol of some vegetables (μg/100g fresh edible portion).

<table>
<thead>
<tr>
<th>Food</th>
<th>β-Carotene</th>
<th>α-Tocopherol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Kale (Sukuma wiki)</td>
<td>4920</td>
<td>4410</td>
</tr>
<tr>
<td>2. Cowpea leaf</td>
<td>7500</td>
<td>6420</td>
</tr>
<tr>
<td>3. Bean leaf</td>
<td>10650</td>
<td>3250</td>
</tr>
<tr>
<td>4. Pumpkin leaf</td>
<td>8500</td>
<td>7530</td>
</tr>
<tr>
<td>5. Onion leaf</td>
<td>3950</td>
<td>3700</td>
</tr>
<tr>
<td>6. Cauliflower leaf</td>
<td>6380</td>
<td>4700</td>
</tr>
<tr>
<td>7. Cauliflower curd</td>
<td>30</td>
<td>150</td>
</tr>
<tr>
<td>8. Amaranthus (Terere) – LL</td>
<td>7600</td>
<td>7040</td>
</tr>
<tr>
<td>9. Amaranthus (Emboga) – SL</td>
<td>7100</td>
<td>6950</td>
</tr>
<tr>
<td>10. Nightshade (dry)</td>
<td>6760</td>
<td>7500</td>
</tr>
<tr>
<td>11. Gynandropsis gynandra</td>
<td>7600</td>
<td>3020</td>
</tr>
<tr>
<td>12. Spinach</td>
<td>3260</td>
<td>4200</td>
</tr>
<tr>
<td>13. Cabbage</td>
<td>50</td>
<td>70</td>
</tr>
<tr>
<td>14. Lettuce</td>
<td>45</td>
<td>230</td>
</tr>
</tbody>
</table>

LL – Large Leaved        SL – Small Leaved
Table 4.4b  Average \( \beta \)-carotene and \( \alpha \)-tocopherol contents of some foods (\( \mu \text{g/100g edible portion} \)).

<table>
<thead>
<tr>
<th>Food</th>
<th>( \beta )-Carotene</th>
<th>( \alpha )-Tocopherol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carrots</td>
<td>5680</td>
<td>310</td>
</tr>
<tr>
<td>Potato (Irish)</td>
<td>200</td>
<td>50</td>
</tr>
<tr>
<td>Onion (stem)</td>
<td>70</td>
<td>280</td>
</tr>
<tr>
<td>Tomato</td>
<td>150</td>
<td>1000</td>
</tr>
<tr>
<td>Banana (Unripe)</td>
<td>150</td>
<td>220</td>
</tr>
<tr>
<td>Banana (Ripe)</td>
<td>100</td>
<td>300</td>
</tr>
<tr>
<td>Green Peas</td>
<td>200</td>
<td>500</td>
</tr>
<tr>
<td>Green grams (dry)</td>
<td>100</td>
<td>120</td>
</tr>
<tr>
<td>Pigeon Peas (Dry)</td>
<td>50</td>
<td>180</td>
</tr>
<tr>
<td>Beans (Dry)</td>
<td>50</td>
<td>140</td>
</tr>
</tbody>
</table>
Table 4.4c Average $\beta$-carotene and $\alpha$-tocopherol contents of seed oils (mg/100g oil).

<table>
<thead>
<tr>
<th>Food</th>
<th>$\beta$-Carotene (mg/100g)</th>
<th>$\alpha$-Tocopherol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Safflower oil</td>
<td>24500</td>
<td>Tr</td>
</tr>
<tr>
<td>2. Corn oil (Elianto)</td>
<td>25300</td>
<td>-</td>
</tr>
<tr>
<td>3. Covo* (refined oil)</td>
<td>13500</td>
<td>-</td>
</tr>
<tr>
<td>4. Neem Seed oil</td>
<td>3560</td>
<td>Tr</td>
</tr>
<tr>
<td>5. Tea Seed oil</td>
<td>1020</td>
<td>-</td>
</tr>
<tr>
<td>6. Orange Seed oil</td>
<td>800</td>
<td>Tr</td>
</tr>
<tr>
<td>7. Erthrina Abyssianica Seed oil</td>
<td>1260</td>
<td>Tr</td>
</tr>
<tr>
<td>8. Custard Apple Seed oil</td>
<td>750</td>
<td>-</td>
</tr>
<tr>
<td>9. Calodendram Capense Seed oil</td>
<td>200</td>
<td>-</td>
</tr>
<tr>
<td>10. Aleurite Meluccana Seed oil</td>
<td>250</td>
<td>-</td>
</tr>
</tbody>
</table>

* - a refined oil from Malawi
Table 4.5 Recovery of added thiamine and riboflavin from some foods

<table>
<thead>
<tr>
<th>Food</th>
<th>Thiamine</th>
<th>Riboflavin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount (µg)</td>
<td>Percentage</td>
</tr>
<tr>
<td>1. Green grams</td>
<td>100.0</td>
<td>88.9</td>
</tr>
<tr>
<td>2. Green peas</td>
<td>150.0</td>
<td>136.0</td>
</tr>
<tr>
<td>3. Beans</td>
<td>150.0</td>
<td>137.0</td>
</tr>
<tr>
<td>4. Spinach</td>
<td>200.0</td>
<td>188.0</td>
</tr>
<tr>
<td>5. Kale</td>
<td>200.0</td>
<td>190.0</td>
</tr>
<tr>
<td>6. Amaranthus</td>
<td>200.0</td>
<td>192.0</td>
</tr>
<tr>
<td>7. Nightshade</td>
<td>200.0</td>
<td>195.0</td>
</tr>
</tbody>
</table>
Table 4.6 Reproducibility results of thiamine and riboflavin in some foods

<table>
<thead>
<tr>
<th></th>
<th>Thiamine&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Riboflavin&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean content</td>
<td>Coefficient of variation (%)</td>
<td>Mean content</td>
</tr>
<tr>
<td>in μg/100g</td>
<td>in μg/100g</td>
<td></td>
</tr>
</tbody>
</table>

1. Green grams  | 696 ± 62.7 | 9.0 | 497 ± 27.7 | 5.6 |
2. Beans        | 598 ± 28.7 | 4.8 | 255 ± 7.9  | 3.1 |
3. Pigeon Peas  | 459 ± 20.1 | 4.4 | 390 ± 15.8 | 4.1 |
4. Kale         | -          | -  | 613 ± 21.7 | 3.5 |
5. Spinach      | -          | -  | 490 ± 25.6 | 5.2 |
6. Amaranthus   | -          | -  | 535 ± 23.5 | 4.4 |

<sup>a</sup> Mean ± SD (N=5)
<table>
<thead>
<tr>
<th>Food</th>
<th>Thiamine</th>
<th>Riboflavin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Spinach</td>
<td>-</td>
<td>0.50</td>
</tr>
<tr>
<td>2. Kale (Sukuma wiki)</td>
<td>-</td>
<td>0.60</td>
</tr>
<tr>
<td>3. Nightshade</td>
<td>-</td>
<td>0.70</td>
</tr>
<tr>
<td>4. Cowpea leaf</td>
<td>-</td>
<td>0.65</td>
</tr>
<tr>
<td>5. Amaranthus (LL)</td>
<td>-</td>
<td>0.50</td>
</tr>
<tr>
<td>6. Onion (leaf)</td>
<td>-</td>
<td>0.25</td>
</tr>
<tr>
<td>7. Onion (stem)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8. Potato (Freeze dried)</td>
<td>-</td>
<td>0.10</td>
</tr>
<tr>
<td>9. Tomato</td>
<td>-</td>
<td>Tr</td>
</tr>
<tr>
<td>10. Banana (unripe)</td>
<td>-</td>
<td>Tr</td>
</tr>
<tr>
<td>11. Green Peas</td>
<td>0.40</td>
<td>0.30</td>
</tr>
<tr>
<td>12. Pigeon peas</td>
<td>0.50</td>
<td>0.40</td>
</tr>
<tr>
<td>13. Beans</td>
<td>0.60</td>
<td>0.20</td>
</tr>
<tr>
<td>14. Green grams</td>
<td>0.70</td>
<td>0.30</td>
</tr>
<tr>
<td>15. Margarine</td>
<td>1.50</td>
<td>3.00</td>
</tr>
<tr>
<td>16. Bornivita</td>
<td>1.90</td>
<td>2.55</td>
</tr>
</tbody>
</table>
Figure 4.01a. Absorption spectrum of α-tocopherol

Figure 4.01b. Absorption spectrum of β-carotene
Figure 4.02. Chromatogram of α-tocopherol and β-carotene standards on a µBondapak C<sub>18</sub> column.

Mobile Phase: Methanol - acetonitrile - chloroform - Water (46:30:18:6)  
Flow Rate: 1.0 ml/min.; chart speed: 0.2 cm/min.; Sensitivity: 0.02 aufs; Detector: uv 297 nm.

(1) α-Tocopherol, (2) β-Carotene.
Figure 4.03. Chromatograms of a carrot extract using same condition as in Figure 4.02 except chromatogram A where the mobile phase was methanol-acetonitrile (60:40).

(1) α-Tocopherol,  (2) β-Carotene,  (3) α-Carotene
(4) Unidentified peaks.
Figure 4.04 Chromatograms of amaranthus and ripe banana extracts.

(1) \( \alpha \)-Tocopherol; (2) \( \beta \)-Carotene, (3), (4) Unidentified Peaks.
Figure 4.05. Chromatograms of Kale and Onion Leaf extracts. Conditions same as in Figure 4.02.

(1) α-Tocopherol  (2) β-Carotene  (3), (4) Unidentified Peaks.
Figure 4.06. Chromatograms of neem seed oil and green peas. Same conditions as in Figure 4.02

(1) α-Tocopherol (2) β-Carotene (3), (4), (5) Unidentified peaks.
Figure 4.07. Calibration curves for β-carotene (1) and α-tocopherol (2)
Figure 4.08. Chromatograms of kale (A) and kale containing added known amounts of pure α-tocopherol and β-carotene (B). Same conditions as in Figure 4.02 except flow rate was 1.5ml/min.

(1) α-Tocopherol (2) β-Carotene (3), (4) Unidentified.
Figure 4.09. Absorption Spectrum of Thiamine
Figure 4.10. Absorption Spectrum of Riboflavin.
Figure 4.11. Chromatogram of green gram extract. Same conditions as in Figure 4.12 were used except the mobile phase was 0.01M phosphate buffer (pH 3.0) - methanol - acetonitrile (80:12:8).

(1) Thiamine, (2) Riboflavin, (3) Unidentified Peaks.
Figure 4.12. Chromatogram of thiamine and riboflavin standards on a µBondapak C₁₈ column. Mobile Phase: 0.01M phosphate buffer + 0.005M 1-hexanesulfonic acid (pH 7.0) - methanol - acetonitrile (80:12:8); Flow Rate: 1.0ml/min.; Chart speed: 0.2 cm/min.; Sensitivity: 0.02 aufs; Detector: UV 265nm.

(1) Riboflavin, (2) Thiamine.
Figure 4.13. Chromatograms of spinach and cowpea extracts. Conditions same as in Figure 4.12.

(1) Riboflavin, (2) Thiamine, (3), (4) Unidentified Peaks.
Figure 4.14. Calibration curves for riboflavin (1) and thiamine (2)
REFERENCES


3. Callow, A. Barbara (1946), Food and Health; Oxford University Press, London.


40. Baker, Herman and Frank, Oscar (1968), Clinical vitaminology: Methods and Interpretations; Interscience Publishers, New York.


67. FAO (1970), Food Composition Table for use in Africa.

1. The concentration of the vitamin of interest was calculated according to the formula:

\[ C' = \frac{(h \times c_s) \times h_s \times (V_f \times 100/w_s)}{h} \]

Where

- \( C' \) = Concentration of the vitamin in the sample in \( \mu \)g per 100g of food
- \( h \) = Peak height of the vitamin in the sample in mm.
- \( h_s \) = Peak height of the standard in mm.
- \( c_s \) = Concentration of the standard in \( \mu \)g/ml.
- \( V_f \) = Volume of the sample extract in ml.
- \( W_s \) = Weight of the sample used for extraction in grams.

100 is a conversion to 100g of food.

2. The standard deviation was calculated using the formula:

\[ S = \sqrt{\frac{\sum (X_i - \bar{X})^2}{n-1}} \]

Where

- \( S \) = Standard deviation
- \( X_i \) = Measured value
\( \bar{X} = \text{Mean value} \)
\( n = \text{Number of tests} \)

3. The coefficient of variation was calculated using the formula

\[
CV = \frac{S}{\bar{X}} \times 100
\]

Where

\( CV = \text{Coefficient of Variation} \)
\( S = \text{Standard deviation} \)
\( \bar{X} = \text{Mean value} \)

4. The linear correlation coefficient was calculated by the formula

\[
r = \frac{(\sum X_i Y_i - (\sum X_i \sum Y_i)/n)}{\sqrt{(\sum X_i^2 - (\sum X_i)^2/n)(\sum Y_i^2 - (\sum Y_i)^2/n)}}
\]

Where

\( r = \text{Simple linear correlation coefficient} \)
\( X_i = \text{Measured value of X} \)
\( Y_i = \text{Measured value of Y} \)
\( n = \text{Number of tests} \)